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Vol. XXXVII. *October, 1919.* *No. 4.*

BIOLOGICAL BULLETIN

NOTES ON THE EFFECT OF X-RADIATION ON THE DEVELOPMENT OF CUMINGIA EGGS.[1]

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The energy of X-rays and of radium rays has been used by a number of investigators during recent years for modifying the normal course of development in living eggs. It has been sought to show by this means what efficacy these rays possess in altering vital properties of protoplasm, and to gain additional information concerning the normal as well as the abnormal reactions of living matter. One of us (Richards, 1914) had studied the effects of X-radiation upon early cleavage and development of *Planorbis*, a gasteropod, and it seemed probable that *Cumingia*, a lamellibranch, would offer interesting comparative data as well as give results of importance in themselves. For this reason the experiment herein described was performed.

Cumingia tellinoides is a form the eggs of which are very frequently used for experimental purposes at Woods Hole. The eggs and sperm are easily obtained separately, and may therefore be subjected to radiation either before or after fertilization.

The normal development of *Cumingia* is not as well known as

[1] During the month of August, 1914, while working at the Marine Biological Laboratory at Woods Hole, the senior writer performed experiments to test the effect of x-radiation upon the eggs of *Cumingia*. This experiment yielded results which would have justified a much more extensive study of the behavior of these eggs under the stimulation of x-radiation, but circumstances have prevented its repetition. In spite of the fact that the experiment has not been followed up, and that the number of eggs worked upon was not large, it has been decided to make note of the results obtained, for they extend our knowledge of radiation effects.

During the course of the experiment eggs were preserved (usually in Bouin's fluid) for later cytological study. This material has been sectioned and studied in the Zoölogical Laboratory of Wabash College.

A. R.

is that of some other forms, but the more important features are described by a number of writers. Morgan, Jordan, Morris and others in their descriptions of experiments have presented normal conditions as well as results of experiments.

In *Cumingia* eggs, Morgan ('10) found that normal development may follow in eggs that have undergone very considerable disturbances during experimental treatment, even "when the visible substances are unequally distributed, and are carried over into the blastomeres, redistribution being thereby prevented." Cleavage is persistently normal under very abnormal conditions, although very many factors may induce abnormal development, and the results manifest themselves only later. Abnormalities are especially apt to be caused by rough handling of the eggs in sea water after laying, injury to the egg membranes apparently being attended with serious consequences. "None of the visible substances are essential to the development of special parts of the embryo."

Polyspermy occurs in *Cumingia* eggs generally unless the sperm suspension be diluted before fertilization. This commonly is the cause of abnormal embryos, if it is permitted to take place to any extent at all. In our experiment the number of extra sperm, while not large, varied definitely depending upon the dosage of the X-rays, and therefore served a useful purpose in giving additional information as to the effectiveness of the rays.

The breeding season of *Cumingia* is at its height late in June and during the month of July. In August it declines and fewer eggs are laid by each female. The general impression prevails also among those who have worked with this form most that at the end of the season the egg is more easily injured, for instance by physical treatment, and so the percentage of eggs of a given lot which develops is less at that time. The number of eggs at any time which will develop depends upon their manipulation, for unnecessary handling with pipettes will cause low ratios.

At the time of fertilization the egg is in the metaphase of the first maturation division. Upon fertilization, maturation is completed in the usual manner.

The form and size of the first cleavage division is very constant and definite. The first division is unequal, the CD cell being

much larger than the AB cell (Morris). The CD cell divides before the AB producing a 3-cell stage, then a 4-cell. After the third division cleavage becomes irregular, the D cell, or its derivatives, remaining for some time distinctly larger than the other blastomeres.

Experiments.

In the set of experiments here recorded, all exposures to the X-ray tube were made at the same time; thus all three-minute irradiations were under the tube simultaneously All three-minute irradiations were removed at the same time, as were all fifteen-minute irradiations. Consequently there is in all the data presented no question whatever as to any varying amount of exposure for the same length of irradiations.

The irradiation intensity was therefore, a constant; hence, we may neglect it entirely and take the time in minutes as a measure of the amount of irradiation, not concerning ourselves quantitatively with the physical units involved. This is a much simpler method since when one attempts to calculate the amount of irradiation he enters a field entirely beyond the scope of this paper. What we wish to show is the comparative effect of different amounts of irradiation when conditions are identical.

Heat, the dilution of the suspension, and the other external factors were kept constant in order to neglect them and reduce any possible chance for error by a faulty technique or varying conditions.

Four series of experiments were set up. In the A series the eggs were irradiated immediately after fertilization; in the B series, sperm were radiated and then used to fertilize normal eggs; in the C series, eggs were radiated and then fertilized by normal sperm; in the D series, both egg and sperm were radiated before fertilization.

The first experiment was carried on to test the effect of short and long irradiations on the freshly fertilized egg. For this test fresh sperm and eggs were obtained from the individual animals and immediately mixed. Fertilization took place immediately. This lot was divided into three parts, one for control and two for experiment. All were allowed to develop under similar conditions. Samples of the three lots were fixed at intervals during

the maturation and early cleavage divisions. A tabulated form of the observations is shown in Table I.

TABLE I.

Time After Irradiation.	Stage of Development.		
	Control.	A3 Radiated 3 Minutes.	A15 Radiated 15 Minutes.
20 minutes.	Completing 1st maturation division.	In second maturation division 1st polar body present.	Beginning 2d maturation division.
43 minutes.	40% in 2-cell stage rest dividing in 1st cleavage division.	Completing 1st cleavage division.	5% in 2-cell stage rest dividing.
60 minutes (1 hour).	36% in 2-cell stage. 14% in 4-cell stage.	25% in 2-cell. 25% in 4-cell.	30% in 2-cell. 10% dividing. 10% in 4-cell.
80 minutes (1⅓ hours).	25% in 2-cell. 17% in 4-cell. 8% in 8-cell.	20% in 2-cell. 20% in 4-cell. 10% in 8-cell.	25% in 2-cell. 20% in 4-cell. 5% in 8-cell.
325 minutes (5 hrs. 25 min.).	17% of those developing are free swimming larvæ.	10% of those developing are free swimming larvæ.	1% of those developing are free swimming larvæ.
385 minutes (6 hrs. 25 min.).	———	———	10% of those developing are free swimming larvæ.

Radiation at first serves to stimulate the mitotic activity of these eggs. The results are not as clear cut as in *Planorbis* where entire egg clusters can be observed without in any way disturbing the eggs in their normal environment. Nevertheless, it is clear here from Table I. that acceleration takes place at first in the irradiated eggs and that subsequent retardation follows as in the case of *Planorbis* where "the control, started at the time of the exposure goes more slowly than the experiment during the first two mitoses, but by the time the twenty-four-cell stage is reached the exposed eggs are progressing more slowly than it" (Richards, '14). Packard ('16) obtained similar results in *Arbacia*.

In a cluster of *Planorbis* eggs division takes place almost simultaneously in all the eggs. No such degree of uniformity is to be observed in *Cumingia*, but the data clearly indicate that the acceleration, while it persists longer than in *Planorbis*, just as surely gives way to a retardation, and that the longer radiation causes a greater retardation than the shorter. It would

appear that greater exposure caused greater stimulation at first than the shorter, agreeing in this particular with the marine eggs which Packard ('16) studied, rather than with the fresh-water *Planorbis* and *Physa*.

From the 43-minute stage on the radiated eggs become slower in divisions, the percentage of free swimming larvæ in A3 being only a little over half the per cent. in the control, 325 minutes after fertilization. Only one or two per cent. of the A15 set have reached the free-swimming larval stage. However, from Table I. we see that one hour later or 385 minutes after fertilization it showed approximately the same percentage as the A3, 385 minutes after fertilization. This shows A15 at 325 minutes after fertilization is 20 per cent. behind A3 and approximately 35 per cent. behind the control in respect to time.

In figuring percentages of gain or loss during development and cleavage, we, following Packard, divide the difference in time required for the two sets to reach a certain stage by the time required by the control to reach that stage. Thus in the A series the control passed the 10 per cent. free-swimming larval stage several minutes ahead of A3. Now A15 entered that stage just 60 minutes later which is 20 per cent. retardation when compared with A3.

It was deemed inadvisable to carry the experiment farther for in both the control and the radiated lots a marked disintegration set in. It has been shown that disintegration may follow exposure to either radium (Packard) or X-rays (Richards, '15), but our radiation is hardly to be held responsible for the results of this experiment, since both control and experiment show it equally.

In Table I. reference is made to the percentage of the entire number of eggs which developed in the experiments. At the height of the breeding season a very large percentage of the number of *Cumingia* eggs fertilized are found to develop, usually over 95 per cent., it is stated. The precaution must be taken in that case to use dilute sperm in order to prevent polyspermy. The eggs used in this experiment, however, were obtained late in the summer and some polyspermy was permitted, for it was found, as will appear later, that the number of sperm entering

the egg is itself influenced by irradiation. A count, made as carefully as possible, of the eggs of this experiment gives 54.25 per cent. as the average number developing. The data given in the tables refer to the number of eggs which developed, not to the total number of the experiment. These numbers were verified by counts of sections as well as of the entire eggs. Of the sections counted 53.5 per cent., and of the whole eggs, 55 per cent. were found to be developing, giving an average of 54.25 per cent.

The B, C and D series were governed by one control. Since all irradiations and fertilizations were at the same time this was made possible. Experiment B was performed to test the effect of two different lengths of exposure when sperm were irradiated and used to fertilize normal eggs. They were subjected to 3- and 15-minute exposure as in the A set. Samples of the control and of the experiment were fixed at varying intervals after fertilization. A study of these samples gave the following results:

TABLE II.

Time After Irradiation.	Stage of Development.		
	Control.	B_3.	B_{15}.
21 minutes.	Completed first maturation division.	Completing 1st maturation division.	Completing 1st maturation division.
31 minutes.	Early prophase of 1st cleavage division.	20% completing 2d maturation divisions and others developing are in the prophase of 1st cleavage division.	Completing 2d maturation division.
74 minutes.	Pro- and metaphase of 1st cleavage division.	Same as control.	Prophase of 1st division.
381 minutes.	20% of those developing are free-swimming larvæ.	25% to 30% of those developing are free-swimming larvæ.	10% of those developing are free swimming larvæ.

From this table we see that 20 minutes after fertilization the first sample shows no difference whatever between control and irradiated sets. The second sample taken 10 minutes later shows no apparent difference yet in the sets. The third sample, 74 minutes after fertilization, shows the short irradiation still the same as the control while the 15-minute irradiation is slightly behind. There is a marked difference in the percentages of

free-swimming larvæ 381 minutes after fertilization. B3 shows an increase, B15 a decrease over the control.

Several observers have noted a stimulation to cell division when eggs have been fertilized by sperm subjected to an exposure of short duration. This observation can now be recorded for *Cumingia*. It is unfortunate that we have no samples of the experiment between the 74-minute and 381-minute stages of development in order to trace this through the cleavage development. The longer irradiation shows the expected retarded development, this length irradiation being harmful.

The C series consisted of radiated eggs fertilized by normal sperm. As stated before the control is the same as for series B. The following is a tabulated result from a study of the samples taken:

TABLE III.

Time After Irradiation.	Stage of Development.		
	Control.	C_3.	C_{15}.
21 minutes.	Completed first maturation division.	Giving off 1st polar body.	Slightly behind C_3.
31 minutes.	Early prophase of 1st cleavage division.	In second maturation division, 1st polar body present.	In second maturation division.
74 minutes.	Pro- and metaphase of 1st cleavage division.	Early prophase of 1st division.	Same as C_3.
381 minutes.	20% of those developing are free-swimming larvæ.	Nearly all dead; only one or two swimming larvæ.	Nearly all dead.

From this table it is evident that even a slight irradiation of the unfertilized egg causes retardation in the rate of cleavage and the development. That they do not develop shows that something has been interfered with in the metabolism of the embryo. Fertilization, however, appears normal in every respect and the polar bodies are given off normally.

The D series consisted of radiated eggs fertilized by radiated sperm. The control was the same as for B. Samples were taken similar to the other series. A study of the samples gave the following results.

Fertilization and maturation are slower in both irradiated sets than in the control. The shorter radiation, 74 minutes after fertilization shows a retardation over the control. The 15 minute

TABLE IV.

Time After Irradiation.	Stage of Development.		
	Control.	D_3.	D_{15}.
21 minutes.	Completed first maturation division.	35% giving off 1st polar body.	Same as D_3.
31 minutes.	Early prophase of 1st cleavage division.	10% in prophase of 1st cleavage division rest completing 2d maturation.	All completing 2d maturation.
74 minutes.	Pro- and metaphase of 1st cleavage division.	Early prophase of 1st cleavage division.	2d polar body present; no prophase of 1st cleavage division.
381 minutes.	20% of those developing are free-swimming larvæ.	Nearly all dead; one third of living are free-swimming larvæ.	Nearly all dead; one sixth of living are free-swimming larvæ.

irradiation is behind the 3-minute exposure. The same handicap is present here as in the two previous series; there being no samples after this period until the free-swimming larval stage is reached. Results of development, 381 minutes after fertilization, however, show that the effect is quite similar to the series where the egg only has been irradiated.

It is worthy of note that here, as in the cases reported by the Hertwigs and by Packard, that the rays produce more effect upon the fertilized eggs than upon the unfertilized. (Compare Tables I. to IV., the A and the B, C, D series.) In general the effect of a short irradiation of the fertilized egg of *Cumingia* is a stimulation in the rate of cell division through the first and second cleavage divisions, after which time there is a retardation. A longer irradiation causes a less acceleration than the shorter treatment and markedly greater retardation and inhibition of growth. In the case of a short irradiation of the sperm (B series) no change whatever can be noted in the rate of division. In the C series where the unfertilized egg is subjected to a short irradiation, the effect after fertilization is a direct retardation of development and a complete inhibition of growth by the time the free-swimming larval stage is reached. There is apparently only a difference of degree in the extent of the injuries due to the two strengths of exposure.

The result of the D series is quite similar to that of the C

series. It is evident that the irradiation of the sperm (B series) is not so harmful as a similar irradiation of the egg.

From a comparison of the number of eggs developing in the different series to that of the control a striking difference is noted. The following table will give by actual count the percentage of eggs developing in the different lots. We find no evidences in the nuclear structure of parthenogenetic development.

TABLE V.

Lot.	Percentage Developing.	Percentage Above Normal.	Percentage Below Normal.
Control (average)	54.25		
B3	60	5.75	
B15	50		4.25
C3	40		14.25
C15	40		14.25
D3	35		19.25
D15	35		19.25

For a sperm exposure of 3 minutes there is a net increase of almost 6 per cent. of the eggs developing. That is, there is a greater number of fertilizations due to a short exposure of the sperm. A longer exposure shows a decrease in the percentage of fertilizations which is below normal. The C series shows a net decrease of 14 per cent., there being no difference evident between the lots of eggs radiated for the longer or the shorter period. The D series is not, as might be expected, an average of the effect of B and C, but is a further decrease, the increase in the B series not being manifested in the D series. In fact the D15 eggs show a deviation from normal nearly equal to the sum of the deviations of the B15 eggs plus that of the C15 lot.

It appears, then, if this single set of observations be regarded as typical, that the percentage of *Cumingia* eggs fertilized is influenced by exposure of the eggs or sperm to X-radiation, and by the amount of exposure which they receive.

In connection with the effect of irradiation upon the percentage of fertilizations is the question of the effect upon polyspermy, for the same factor which would increase the percentage of fertilization would also tend to increase the number of supernumerary sperm to enter the egg. The results of actual counts

of the number of extra sperm in the sections studied are presented in Table VI.

TABLE VI.

Lot.	Percentage Developing.	Extra Sperm.	No. Above Control.	No. Below Control.
Control (average)	54.25	1.59		
B3 (average)	60	3.40	1.89	
B15	50	1.20		.39
C3	40	2.58	.99	
C15	40	1.46		.13
D3[1]	35	1.00		.59
D15	35	1.50		.09

Fertilization, of course, is not simply a selection by the egg of one sperm nucleus out of several, for if that were the case it would follow that the increase in the number of sperm to enter the egg would insure a higher percentage of fertilizations. There is no definite relation between the number of eggs fertilized and the number with more than one sperm. B3 has the greatest number of extra sperm, and is also the highest in number developing; C3, however, is next in number of extra sperm, but the percentage of the eggs in development is below the control. These results show, since columns one and two of this table are inconsistent with each other, that at least one other factor (and, of course, many factors) are involved in fertilization which play no part in polyspermy. In the latter case the factors which cause the entrance of the sperm are the ones involved.

In interpretating the data collected in Table VI. care must be exercised. In the first place it is not possible to control the concentration of the sperm so that relatively many more may not have been added to C3 eggs, for example, than to D3. It is also possible that one set may have been handled more than another, although precautions were taken to eliminate these sources of confusion. Previous investigators have found a great deal of variation in the responses of different lots of *Cumingia* sperm and eggs during development, and the factors governing the behavior of any given lot are rather elusive and uncertain. It has already been pointed out in this paper that these experiments should be carefully repeated before a final acceptance of the interpretations here given.

[1] Number of eggs counted was too small to permit an entirely trustworthy observation as to polyspermy.

The behavior of the extra sperm in the experiment, however, calls to mind the change in the activity of *Arbacia* fertilizin under the influence of X-radiation (Richards and Woodward, 1915). This secretion from *Arbacia* eggs possesses the power of activating sperm, and this property is affected by X-rays as follows: a slight radiation (2 min.) increases its activity, a long one (7 min.) lessens it, while an exposure of intermediate duration is practically without effect. Since a solution of *Arbacia* fertilizin in sea water (egg water) is definitely affected by irradiation, it is very probable that the substance which escapes from the eggs into the water would also be influenced by the irradiation before it passes out of the eggs.

The egg secretions of *Cumingia* and their properties have not been investigated, but Miss Cobb (see Woodward, '18, p. 464) "discovered that the eggs and egg water of *Cumingia* produce positive chemotactic response on the part of the sperm." Miss Sampson has found (unpublished) a secretion from *Cumingia* eggs which agglutinates its own sperm into very small groups of three of four sperm, and produces a very strong agglutination of *Arbacia* sperm.

In view of these facts and reasoning from the analogy, we are tempted to explain the facts shown in Table VI. by assuming that the fertilizin of *Cumingia* while yet in the egg was affected by the irradiation in such a manner as to bring about the results shown. The long radiations in each case where eggs were exposed, C15, and D15, show fewer extra sperm than the control which is to be expected if the long exposure decreases the activity of the substance as in the case of *Arbacia* fertilizin. Likewise in C3, a short exposure, more sperm entered into than the control eggs. This, too, is to be expected if the short irradiation activates the fertilizin. D3 represents the count of too few eggs to be dependable. In B3 and B15 where the sperm were radiated with the result that an increased number entered the egg after the short exposure and fewer than the control number are present, we may be dealing with a similar effect of the irradiation upon the sperm receptors (see Lillie, '19), although at the present time there is not even an analogy upon which to base such an assumption.

The irradiation of *Nereis* eggs (Packard) before fertilization frequently caused marked polyspermy, due to interference with the jelly formation about the egg. In *Nereis* the amount of jelly extruded from the egg at the time of fertilization is characteristically large; a similar explanation here is doubtfully possible in view of the smaller amount of jelly.

There is also other evidence that fertilization is affected by exposure to X-rays by means of a chemical change in the combining substance in the egg or the sperm. Richards ('15) and Packard ('16) have both shown the power of a short radiation in effecting a chemical change in proteids and some enzymes. Swartz ('08) found that exposure to radium is able to effect a chemical change in lethicin. Hertwig ('14) showed the direct effect of radiations of radium upon the chromatin content of the cell. These instances all serve to render plausible the explanation here suggested.

Summary.

1. A short radiation of the fertilized egg of *Cumingia* stimulates cell division for a time then retards it. A longer radiation produces less stimulation and a greater retardation.

2. A short or long sperm radiation before fertilization if not too intense affects the rate of cleavage in no way.

3. A short radiation of the unfertilized egg causes, when fertilized with normal sperm, a retardation in development, the production of abnormalities and complete inhibition of growth before the free-swimming larval stages are reached.

4. A short irradiation of both sperm and egg before fertilization results in a direct retarding of cleavage and in the production of abnormal embryos, most of them never reaching the free-swimming larval stage.

5. Eggs fertilized by sperm that have been subjected to a short exposure to X-rays result in a greater percentage of developing eggs than would normally occur. A long radiation produces an indifferent result.

6. An exposure to X-rays of the unfertilized egg results in a lesser percentage of developing eggs upon fertilization by normal sperm. No difference is evident due to time of exposure.

7. Eggs and sperm each exposed to the X-ray before mixing

results in a lesser percentage of developing eggs than when the eggs alone were irradiated before fertilization. No difference is evident due to time of exposure.

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THE VAGINAL CLOSURE MEMBRANE, COPULATION, AND THE VAGINAL PLUG IN THE GUINEA-PIG, WITH FURTHER CONSIDERATIONS OF THE ŒSTROUS RHYTHM.

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Two years ago we recorded the results of a detailed study of the œstrous cycle in the guinea-pig. A rather full description of the histological and physiological changes which take place in the ovary, uterus and vagina during the "heat period" was presented. We emphasized particularly the importance of changes occurring in the microscopic composition of the vaginal fluid as indicative of the exact conditions in the uterine wall and ovarian follicles at corresponding moments.

Since that time we have somewhat extended the analysis of these phenomena. It has been found that a membrane covering the orifice of the vagina furnishes a most valuable and simple means of diagnosing certain periods in the œstrous cycle. This we have termed the "vaginal closure membrane." The exact moment of copulation and the conditions in the walls of the vagina and uterus at this time have been carefully followed, along with a consideration of the formation and significance of the vaginal plug. In the present paper a discussion of these several topics will be undertaken.

Certain points in the literature will also be discussed, a more complete review having been given in the previous article.

1. THE VAGINAL CLOSURE MEMBRANE.

In the former communication attention was called to the fact that "the external vaginal orifice, which during the period of œstrous activity is more or less open, actually showing in many cases a little fluid or some blood, closes and becomes less accessible after the period." During ovulation the vagina is open, but the fact of its being open is not unmistakable proof of the time of

ovulation unless the open vagina also contains what was described as second or early third stage œstrous fluid.

At that time the method of closure of the vagina following the œstrus was not explained nor was its actual significance fully appreciated. The vagina is now found to be closed by a remarkable cellular membrane and in a very definite way.

The external orifice of the vagina is crescentic in shape and the urethral opening lies in front of it in the mid line. The anterior and posterior lips of the crescent-shaped opening come together, and a delicate epithelial membrane grows over the opening and unites the lips. This occurs shortly after the heat period in females that have not copulated and in those that have copulated the closure follows the expulsion of the vaginal plug, a process to be considered beyond. The closure begins at the tips of the crescent-shaped opening and progresses toward the midpoint. The lips do not approximate so intimately at the midpoint and the membrane here seems to be under more tension than at other parts, even after the entire orifice has closed. The opening of the orifice by a tearing of the epithelial membrane begins at the strained middle part and extends from there laterally until finally the vaginal lips are freely separated. The midpoint is, therefore, the last to close, and the first to open as a general rule, although at times the opening may begin at either side of the midline.

The epithelium completely unites the lips of the vagina so that nothing can escape from or enter into the vaginal lumen without tearing this closure membrane. Such a membranous closure of the vaginal orifice is unknown to us in any other mammal. In many species the sides of the vaginal opening may be approximated or cemented together by some hardened fluid or secretion so that the lips are not readily pressed apart, but a membranous growth closing the orifice after each heat period is apparently unique.

This membrane also completely closes the vaginal opening throughout pregnancy and only becomes ruptured when the vulva swells shortly before parturition.

Such an obstruction or closure of the vaginal lumen at once suggests the hymen of the human vagina. But this, of course,

is quite a different structure in its origin as well as in its later history. The closure membrane in the guinea-pig not only exists in the young immature animal but is regularly destroyed before and reformed after every heat period that takes place during the life of the female. The formation or growth of this membrane might also be compared in some respects to the membranous growths tending to extend across and close the pharynx and other canals under pathological conditions.

The membrane is thin and delivate in structure and when stretched by slightly pressing apart the lips of the vagina with the fingers it is seen to be almost transparent, the outline of the vaginal lumen showing through. The closure membrane is of the same glossy appearance as is the surface epithelium covering the region of the vaginal lips with which it is continuous. It is composed simply of stratified squamous epithelium which has grown from the borders of the lips over the orifice and contains no vessels or blood.

When the membrane is torn or broken by accident during the diœstrum, or period of sexual rest, it reforms sometimes within a day, or within a few days, and remains until the beginning of the new period of heat or œstrus. A recognition of this membrane is then a great convenience in determining the onset of the œstrus in a group of female guinea-pigs. Daily smears of the vaginal fluid are not now necessary to find when the œstrus is about to begin in animals examined for the first time and whose rhythm is therefore unknown.

Although the presence of the closure membrane is a definite aid in recognizing the condition of the œstrous cycle, it must be remembered that this membrane often persists up to the first stage of œstrus, at which time the lumen of the vagina is filled with a mucous fluid and first stage cells. This is actually the "heat" time and the normal moment for copulation as we shall explain below. When the closure membrane still persists until the vaginal lumen is so filled it may be distended and rounded out resembling a blister membrane on the point of bursting. Puncturing this the vaginal fluid oozes out through the break. As a general rule, however, the vulva becomes inflamed and very slightly swollen immediately before œstrus and the stretched

membrane breaks. Thus the membrane has reached the breaking point or has actually broken at just about the time the female is in heat and ready to copulate.

While the presence of this membrane is a reliable index of the œstrous condition, the open vagina, or its absence, is by no means indicative of the œstrous state. Although the vagina is always open during what we have termed the second and third stages of œstrous, and, therefore, at the time of ovulation whether copulation has taken place during the first stage or not, it is nevertheless frequently open at other times. It is not permissible to assume that the open vagina indicates a state of heat or the time of ovulation in a guinea-pig. Only when the open vagina contains fluid showing on examination the cells described as second or early third stage is the ovary almost exactly in the condition of ovulation. It may be stated parenthetically that after long experience one is able as a rule to diagnose the stages of the vaginal fluid by slight difference in color and consistency without microscopic examination.

Finally, then, when the vagina is open one may only be certain of the uterine and ovarian conditions by examining the contents of its lumen, but, on the other hand, if it be closed by this membrane one may be certain that the time of the new ovulation has not yet arrived.

2. The Time and Manner of Copulation and the Conditions in the Reproductive Organs of the Female at this Moment.

It is well known that female guinea-pigs in common with other animals of their class, and in fact most mammals, have a definite limited time during which they accept the male, the so-called "period of heat." This period, very slightly revealed by external signs at the mouth of the vagina, but chiefly by the act of copulation has been the starting point in all previous studies on the reproductive activities of the guinea-pig. In order to prevent the modifying conditions of pregnancy following copulation, various operations have been resorted to, as in the case of some of Loeb's experiments. Such operations might complicate or even vitiate the results which follow.

In the present account we wish to describe the exact moment at which copulation takes place during the sexual cycle and to show definitely the conditions of the vagina and uterus at this moment. From the condition of the vagina or uterus the ovarian condition is readily estimated, as we have shown in the former paper. After determining the exact œstrous condition of a female at the moment she is ready for copulation we may then recognize a corresponding moment in any female by an examination of the vagina without the necessity of introducing the male or permitting copulation to occur.

In order to designate the copulation time exactly, we must review briefly the characteristics of the four very clearly defined stages of the œstrus or "heat period" proper. During stage one the uterine epithelium swells, the cells becoming distended with an abundant mucous secretion which very soon pours into the lumen and reaches the vagina. At this time a desquamation of the epithelial cells from the lower part of the vagina also begins. The second stage shows a great accumulation of leucocytes below the uterine and vaginal epithelium with a slowly progressing desquamation of epithelial cells. The third is the stage of exodus of the leucocytes, myriads of them coming through the epithelial lining of the walls of the uterus and vagina, with an accompanying extensive destruction of the epithelium. During the fourth stage the broken down epithelium falls away in masses and at the same time a regeneration of epithelium takes place beginning from the mucosa of the uterine glands.

The Graafian follicles of the ovary rupture and ovulation occurs at the end of the second stage or the beginning of the third stage, while during the fourth stage the recently ruptured Graafian follicles are already well under way in their development into new corpora lutea.

A recent abstract by Long ('19) seems to indicate that four similar stages may be recognized during the œstrus in the rat, and that these stages agree almost exactly in significance with the comparable ones in the guinea-pig: Ovulation also occurring in the rat at the end of the second or beginning of the third stage.

During our initial investigation we made no attempt to locate the exact moment of copulation and, of course, did not describe

the corresponding vaginal or uterine stage. The description by Loeb ('14), however, of the great leucocyte migration in the wall of the uterus twelve to twenty-four hours after copulation would indicate that the true "heat" or copulation occurs before the beginning of the destructive changes in the uterus, and this we now find to be true. Here and in the following section we wish to point out just how the uterine changes seem to be associated with the act of copulation, the retention of the sperm in order to insure the fertilization of the eggs, and after this the means of ridding the vagina of the excessive seminal accumulation.

A number of females have been placed with males while in one or another of the above mentioned four stages, as well as during different times of the diœstrum, or interval of sexual rest. The results show that a copulation is never accomplished except during the first stage of œstrus about twelve hours before the second stage begins. Long ('19), also finds copulation to take place in the rat during the first stage. At this stage in the guinea-pig the vagina contains a clear, foamy, saliva-like fluid in which desquamated epithelial cells of the first type are present. Differing from all other stages and times there are now no leucocytes to be found in the vaginal fluid,—compare our former Figs. 1 and 2 with Figs. 5 and 6. Even during the resting period the vagina contains some mucus but this is very scant and filled with many leucocytes, being pus-like in appearance and consistency.

To locate even more accurately the normal time of copulation the first stage may be subdivided into two shorter periods: a preparatory interval, the early beginning of the first stage, when the vagina is almost dry and contains only a scant amount of loose cells of the first type; and, second, what may be designated the true first stage, a more advanced period when the frothy mucous secretion has already begun to accumulate. Copulation takes place during this second phase of stage one and never during the first.

During stage one the vagina is characterized by two important conditions, both of which contribute to the success of copulation. In the first place the existence of a mucous secretion evidently

facilitates the act of copulation. This abundant frothy mucous accumulation is limited to the first stage, particularly to the time when copulation takes place, and it never occurs in the vagina at other times. The second contributory condition is the complete absence of leucocytes in the mucous fluid. The leucocytes begin their migration through the epithelium of the uterus and vagina at the end of the second stage. They are extremely abundant in the lumen during the third stage, while from this time on they are found in the uterus and vagina in smaller or larger quantities up to the approach of the next first stage. Two days before the first stage begins leucocytes are still plentiful, but from this time first stage epithelial cells gradually become more abundant and the leucocytes decrease in number until finally when the first stage has actually arrived no leucocyte exists in the vaginal fluid. The mucous content of the vagina during the first stage hence lacks the pus-like appearance of the vaginal fluid of the "intermenstrual" time and is clear and foamy.

The absence of leucocytes from the vaginal lumen at the time of copulation is important, since, if present, they might by their dissolving powers or phagocytic action exert an injurious effect on the spermatozoa and thus interfere with their normal function. Later a special purpose of the leucocytes seems to be to destroy the excess of spermatozoa remaining in the uterus. This frequently occurs by an interesting process of phagocytosis. A leucocyte comes in contact with a spermatozoön which with its tail is longer than the leucocyte. The leucocyte by stretching and contracting finally takes into itself the entire spermatozoön, the tail being wound in circular fashion within the cell body. The leucocytes, however, apparently accomplish most destruction by their dissolving or disintegrating action.

It seems that the migration of the leucocytes through the walls of the uterus and vagina, though not increased in extent, is accelerated by the act of copulation and the entire œstrous process is shorter than in non-copulated females. About six hours after copulation the third stage is in full development, while under virgin conditions a comparable stage is reached only after at least twelve hours from the time when copulation might have occurred. It may be said that copulation tends to

hasten ovulation, or that the act itself may facilitate the bursting of the Graafian follicles, which is a very old conception.

The act of copulation is short, lasting a few seconds only, while the preceding time of sexual excitement leading up to it is rather long. The male becomes excited by the presence of the female some time before she reaches the proper condition for copulation. A male after long isolation from females becomes sexually excited by the presence of any female irrespective or her sexual condition, and he invariably attempts to copulate. Nevertheless, the excitement of the male is not so strong nor prolonged when in the company of a female during sexual inactivity as with one during her sexual season. When the female is nearing œstrus the male is extremely excited and tries again and again to copulate, while at other times he soon tires and loses interest and ceases his aggressive behavior.

The male and female never fight during the long period of aggressiveness on the part of the male, which often lasts for many hours. The male tries to induce the female to copulate by irritation and excitement rather than by forcing her. The female may at times become nervous and attempt to bite the male, but an actual fight such as occasionally occurs between two males never takes place. No mating by force is observed; the consent of the female is necessary for the completion of copulation. Copulation is followed by a state of relaxation similar to that observed among mammals in general, and immediately afterwards both male and female may spend some time in cleaning their external genitalia.

3. The Vaginal Plug, Its Formation, Length of Existence and Manner of Discharge.

The spermatic fluid of the guinea-pig, especially that portion derived from the seminal vesicles, on entering the vagina of the female coagulates to form the *bouchon vaginal*, a rigid plug, filling the lumen of the vagina. This plug prevents the outflow of the sperm after every copulation. Such a vaginal plug has been described in many species of rodents and seems in general to be characteristic of this class of mammals. It was first observed in the guinea-pig by Leuckart in 1847. He correctly

described it as a Pfropf (plug), formed by the coagulation of the secretion from the seminal vesicles and serving to fill the vagina and prevent the flowing out of the sperm after copulation.

Bischoff, in 1852, verified the observations of Leuckart and accepted his conclusions regarding the rôle of the vaginal plug in the copulation process. Reichert, 1861, differed with these two original descriptions in failing to find the formation of a vaginal plug after every copulation, and concluded that its presence was not a general phenomenon. Later, however, Hensen in 1876 brought new evidence confirming the observations of Leuckart and Bischoff.

Landwehr, in 1880, examined the seminal vesicles and found their secretion to contain twenty-seven per cent. of fibrinogen to which its coagulation reaction is due. Coagulation may occur as soon as the secretion of the seminal vesicles comes in contact with a small amount of blood.

Héron-Royer, 1881, observed the vaginal plug in *Pachyuromys duprasi*, but gave no satisfactory explanation of its formation. According to him the vaginal plug was formed in the vagina before copulation and was pulled out or loosened by the hooks on the penis during the act of copulation. These observations were entirely contrary to all earlier records, according to which the plug is formed after copulation and falls out some hours later. Blanchard made histological examinations of the vaginal plugs collected by Héron-Royer and found them to consist of two parts, a central, *partie centrale*, composed chiefly of great numbers of spermatozoa, and a peripheral part, *couche corticale*, formed of hardened mucus.

Lataste, in 1882, after examinations of the vaginal plug in the same species, *Pachyuromys duprasi*, came to quite different conclusions. He states that the vaginal plug, *bouchon vaginal*, as he termed it, is not formed as Héron-Royer claimed, before copulation, but immediately after, and in the same way as was known for other rodents. Regarding its function he accepted the old opinion of Leuckart that it serves to prevent the spermatozoa from flowing out of the vagina after copulation. He also mentions an instance in which a vaginal plug-like formation was found when there had been no previous copulation. From our

observation on the œstrous discharge in the guinea-pig it is probable that this plug-like structure was nothing else than a concentrated accumulation of such a discharge, it having become unusually dense or dried out. In fact, as will be shown beyond, the superficial portion of the vaginal plug is actually the sluffed-off vaginal epithelium surrounding the coagulated seminal fluid. Thus the plug is partly of vaginal origin.

In later papers Lataste makes many contributions to the knowledge of the vaginal plug. In 1883 he described the vaginal plug in other rodents and pointed out that this formation was evidently not limited to a few species but was characteristic of the entire class.

Regarding the function of the vaginal plug, he slightly modifies his former position and concludes that its rôle is not only to prevent the sperm from flowing out of the vagina but rather by a filling up to push the sperm into the uterus. He extended the observation of Blanchard that the vaginal plug consists of two parts, differing in structure, a central core and a superficial envelope. He described the central part as consisting chiefly of the coagulated secretion of the seminal vesicles and also of a quantity of mucus, 1888*a*, while the superficial portion, *enveloppe vaginale*, was formed of stratified epithelial cells. The *enveloppe vaginale* is produced in the female by a rapid exfoliation of cells from the uterine glands and the vaginal walls on account of the irritating presence of the coagulated core. (His conception of the cause of the exfoliation is entirely incorrect.) The envelopment of the core by loosened epithelium from the vaginal wall serves to make easy the expulsion of the vaginal plug. This epithelial production he thinks is probably of a pathological nature and may be compared to the condition in women known as *vaginite exfoliante*.

These studies of more than thirty years ago by Lataste are in most respects surprisingly correct and it is only the nature of the process by which the outer epithelial envelope is formed with which we would materially differ.

Tafani, in 1888, described the vaginal plug in the mouse and found it to fall out about thirty hours after copulation.

Steinach ('94) found that the removal of the seminal vesicles

from rats did not influence their sexual instincts or ability to copulate, but decidedly impaired the power of the male to fertilize the female.

Sobotta ('95) also has studied the formation of the vaginal plug in the mouse and found it present after every copulation. Histologically it consists of an homogeneous mass which is surrounded by an envelope of vaginal epithelium. Spermatozoa are more abundant in the central mass at its upper end or that portion near the uterus as the plug lies in the vagina. He confirms the observations of Tafani regarding the fate of the vaginal plug, finding that its surface gradually becomes soft and loose and the entire mass falls out of the vagina about twenty to thirty hours after copulation. Sobotta states that the vaginal plug in the guinea-pig falls out much sooner than in the mouse, being eliminated from the vagina within from four to nine and a half hours after copulation. The longer interval is approximately correct. He claims to have at times observed another copulation following the expulsion of the first vaginal plug.

Camus and Gley ('96) studied the coagulation process in the formation of the vaginal plug. They claim coagulation to be due to the influence of a prostatic enzyme, "vesiculase," upon the secretion of the seminal vesicles. The action of the prostatic enzyme is specific towards the seminal vesicle secretion of any rodent. The prostatic enzyme of a rat will coagulate the seminal fluid of a guinea-pig and vice versa.

Rubaschkin ('05) returns to the old opinion of Reichert, 1861, in claiming that the vaginal plug is not a constant formation in the guinea-pig following copulation. His statements are as follows: Bei der Maus (Sobotta), und nach Bischoff und Hensen auch bei Meerschweinchen bildet sich nach dem Coitus ein charakteristischer Vaginal-pfropf, der auf einen vorausgegangenen Coitus hinweist. Ich muss hier die Beobachtungen von Reichert bestätigen, dass beim Meerschweinschen ein solcher Vaginalpfropf sich meistens nich bildet. Von aussen konnte ich einen klaren Pfropf in der Vagina niemals erkennen; in einigen Fällen liessen sich einige Schleimstreifen bemerken, die aber ganz unregelmässig und nicht immer zu Tage traten. In seltenen Fällen wurden nach dem Secieren Vaginalpfröpfe gefunden,

welche zum Teil aus verdichtetem Schleim, zum Teil aus Epithelzellen bestanden. Unter diesen Verhältnissen ist die Bedeutung des Vaginalpfropfs beim Meerschweinchen ganz nichtig, und am Anfange meiner Arbeit habe ich, durch diese Angabe Bischoff's irregeführt, einige Tiere verloren, weil sie zu spät getötet wurden.

Königstein ('07) described the vaginal plug in rats and agrees with the observations of Lataste, Tafani and Sobotta. He finds also the vaginal plug to consist of two parts, a central and a superficial. The vaginal plug contains in addition to the secretions of the male genital glands, mucus, detritus, many leucocytes, squamous epithelial cells in large numbers and a granular eosinphil staining secretion.

From this review the knowledge of the formation of the vaginal plug is found to be rather complete, although disagreements as to facts are expressed by several authors. It seems well established that the formation of a vaginal plug following copulation is a general phenomenon among the various species of rodents. The plug proper consists of a central core formed mainly by coagulated fluid from the seminal vesicles and this is surrounded or enclosed by a mass of flat epithelial cells, apparently derived from the vaginal wall. The coagulation of the seminal fluid may be due to the action of a prostatic enzyme although it is claimed that the coagulation occurs without the presence of such an enzyme. The vaginal plug as a whole falls out of the vagina a few hours after its formation.

On the other hand it is not clear from the literature just how or why the peripheral part of the vaginal plug, *enveloppe vaginale*, of Lataste is formed. And the manner and cause of the separation of the epithelial lining from the wall of the vagina are also unknown. These points could not be clearly understood without a knowledge of the changes occurring in the wall of the vagina and uterus during the œstrus, at which time copulation and the formation of the vaginal plug take place.

As we pointed out in our description of the œstrous changes, there is a stage in the cycle when immense numbers of leucocytes accumulate immediately below the epithelium lining the uterus and the vagina. From this position the leucocytes attack the epithelial cells and at the same time dissolve or destroy the

connection between the mucosa and the subjacent connective tissue over extensive areas. This reaction is taking place a few hours after copulation during the latter part of stage one and throughout stage two of our description. A few hours later, during stage three, the leucocytes have made still further progress in their invasion of the mucosa and the destruction of its connection with underlying tissues. In certain sections of the uterus the entire mucosa filled with immense numbers of leucocytes is completely separated from the uterine wall and lies within the lumen, while in other regions the epithelium is loosely connected but still hanging to the wall. This disconnected and degenerating mucosa loaded with leucocytes breaks into small fragments during the fourth stage and is expelled from the uterus and vagina, while a new mucosa begins to regenerate from the mouths and the regions about the uterine glands and from the deeper layers of the vaginal epithelium. This is the fate of the mucosa when no copulation has taken place.

There is, then, no pathological "vaginite exfoliante" due to an irritation of the vaginal wall by the seminal fluid as Lataste thought. But a simple periodic œstrous breaking down of the uterine wall under leucocyte invasion, entirely independent of whether copulation takes place or not.

When copulation has occurred the loss of the epithelium follows a somewhat different course. Immediately after copulation the coagulated seminal fluid forms a mass within the lumen of the vagina and partly extending into the uterus. Around this mass the mucosa forms a close fitting envelope, thus preventing its early dislocation. The envelope serves to retain the plug in the vagina until the fourth stage of the œstrous cycle at which time the enveloping epithelium becomes completely separated from the vaginal wall by the dissolving effects of the leucocytes. The epithelium is now expelled as one continuous tube forming the cover around the vaginal plug instead of sluffing off in smaller pieces as occurs during the fourth stage when a copulation has not occurred. However, the vaginal epithelium may occasionally be shed en masse without copulation. In one striking case the epithelium was pulled out of the vagina as a conical sheath, enclosing the speculum that had been introduced for examination.

It is clear, therefore, that what was termed by Lataste the "enveloppe vaginale" is the layer of epithelium separated from the underlying connective tissue by the dissolving action of the leucocytes which invade the walls of the uterus and vagina at this time. It is also readily understood how the plug, after its short sojourn in the vagina and cervix of the uterus, is finally separated from its adhesion or tight connection with the wall and expelled as a mass from the vagina.

A possible function or effect of the vaginal plug in addition to those before mentioned has recently been suggested by Long ('19). He states that a stimulation of the cervix of the uterus in rats, by merely inserting a glass rod during stage one of the œstrus, prolongs the next cycle, and suggests that the vaginal plug may also act in this mechanical way. We have not tested the prolongation of the cycle in guinea-pigs following copulation without conception as compared with its length in virgin animals.

4. The Œstrous Rhythm.

In our earlier review of literature it was pointed out that the knowledge of the actual time of ovulation in the guinea-pig was decidedly inexact. Nothing scarcely was known of the periodic recurrence of the œstrus stages in a given female. In short the moment of ovulation in the guinea-pig was not available for accurate experimental purposes and no definite criterion or method had been devised for detecting the œstrous condition. And this was true in spite of a very long list of studies pertaining to the reproductive activities of these animals.

Reichert, as long ago as 1861, had found that the Graafian follicles rupture about nine to ten hours after copulation. This, in general, approaches correctness, but in cases where copulation has not taken place, or failed to be observed, such knowledge is of little consequence. Rubasckhin ('05) had more recently claimed that the vagina was open and the vulva somewhat inflamed ten to twelve days after parturition, but this is certainly too short an interval to indicate an actual return of heat. It must be remembered that the female guinea-pig goes into "heat" and accepts the male almost immediately after the delivery of her litter. This fact makes the length of Rubaschkin's interval still more improbable.

The most valuable and extensive investigations of the reproductive activities of the guinea-pig were those made by Leo Loeb ('11, '14). But here the data were derived almost entirely from examinations of the uterus and ovaries after their removal from the body of the female. While such studies did give a means of comparing the conditions found among different individuals at different times, and made it possible to estimate approximately the length of the sexual periods, yet this estimate could not be transferred with certainty to any one living individual. We further objected to Loeb's method of study since it failed to permit an investigation of the recurring œstrous periods in a number of unoperated females. The results of such an investigation would be most important in determining the influence of any unusual or experimental conditions introduced with intent to modify the intervals between ovulations or other periods of the sexual cycle. These are just such problems as Loeb had under consideration.

The entire literature showed that any such thing as a regular œstrous flow was completely undiscovered for the guinea-pig. It became necessary, however, for our studies to have an accurate knowledge of ovulation times, and to determine this, extensive investigations of the sexual cycle in the guinea-pig were undertaken. A simple method of examining the vagina of the living animal proved to be of the greatest value. Virgin females were selected and the fluid present in the vaginæ was taken daily by means of a small nasal speculum and cotton swab. This fluid was smeared on slides, stained and studied microscopically. The method is fully described in the former paper.

It very soon became evident that the vagina generally contained little or no fluid, but that periodically a great accumulation of mucus and cells was to be found. This excessive amount of mucus and cells is to be recognized as a typical œstrous flow. The constituent elements of the fluid change in their relative abundance in a definite manner from the beginning to the cessation of the flow. Four clearly marked stages, as mentioned above, could be separated by microscopic examination of the fluid smears.

These changes in the composition of the vaginal fluid were

found to be associated with comparable changes in the structure of the epithelial walls of the uterus and vagina. And not only was this the case, but the changes in the vaginal fluid proved to be most reliable indices of definite processes taking place in the ovaries in connection with the rupture of the Graafian follicles and the expulsion of the ova. It is, therefore, evident that by an examination from time to time of this fluid, one may know the exact condition of the ripening follicles in the ovary and very nearly the exact moment of ovulation.

The œstrous cycles in a group of guinea-pigs were followed for a number of months in order to establish the normal periodicity or rhythm. The amount of variation that might exist in the length of the cycles in a given female was studied as well as the variations in cycle lengths among different individuals. An attempt was further made to discover any seasonal variations that might exist.

Only slight time variations were found in the periodic rhythm of a given female. For example, in one animal the record of six consecutive periods shows the œstrous flow to begin on the sixteenth day five times and on the fifteenth day once. In another case of seven consecutive periods the flow began on the sixteenth day six times and on the seventeenth day once. For further cases the reader is referred to the table given in our former paper.

There is only a limited variation in the length of the œstrous cycles among different individuals, ranging between fifteen and seventeen days in younger animals. In exceptional cases the period is slightly lengthened in older multiparæ, sometimes reaching eighteen days. These limits of fifteen and eighteen days for the lengths of the œstrous cycles have never been violated under normal conditions during the several hundred observations which we have now recorded. The method of examining the vagina for the closure membrane above described, and, in the case of its rupture, for the composition of the fluid contained within the lumen, renders these individual variations of no consequence in determining the exact "heat period" and time of ovulation.

Slight, if any, seasonal variations are shown by our animals. This may be due, however, to the uniformly warm temperature maintained in the breeding rooms during the winter months.

For a full description and photographs of the structural changes occurring in the œstrous fluid, the vagina, uterus and ovaries, the reader is referred to the original account.

After the publication of our results it was found that one of the last papers by Leo Loeb ('14), bearing on a related subject, had unfortunately been overlooked. We regret this, since a discussion of his methods and results would have been somewhat clearer in connection with our full consideration of the œstrus given in the previous paper than in the present connection. In earlier papers Loeb ('11) had completely failed to establish a definite length or periodicity for the sexual cycle in the guinea-pig. In the last paper, however, the length of the cycle was more nearly determined and a very thorough description was given of the microscopic changes taking place in the uterine wall during the heat period. Our independent account of the structural changes in the uterine wall fully confirms Loeb's description. But we are unable to agree exactly with the lengths of the sexual periods as estimated from his examinations of the removed uteri. In a still more recent article Loeb ('18) repeats his 1914 estimates and claims the lengths of the sexual cycles to vary between thirteen and a half and nineteen days.

In all cases Loeb's investigations had centered in a study of the sectioned uterus and ovary, thus necessitating their removal by operation or the death of the animal. Either procedure permits only one observation on a given female. No investigation of the uterus and vagina in the living animal had been made and no continuous observations on the consecutive cycles of given individuals were carried out.

As mentioned before, we recorded not only the structural changes of the uterus, but almost equally as marked changes in the wall of the vagina. And what we consider to be of still more importance from an analytical or experimental standpoint as a means of estimating the moment of ovulation, was the complete record of the changes in the microscopic composition of the vaginal fluid during the different stages of the sexual cycle. The removal and examination of this fluid is made without in any degree injuring the uterus or vagina and does not interfere with the further use of the female for ovulation and breeding

records. This knowledge of the definitely changing structure of the vaginal fluid made it possible to study the œstrous cycles in many living females and reduced the time element of ovulation in the guinea-pig to a certainty.

We considered in a somewhat different manner the connection between the uterine reaction and the secretion of the corpora lutea, though essentially we share Loeb's ideas of the functions of these bodies. It was concluded that the duties of the corpora lutea are probably about what Beard ('97) long ago argued in his monograph on "The Span of Gestation and the Cause of Birth."

The development and the degeneration of the vaginal and uterine mucosæ were found to follow very closely the development and degeneration of the corpora lutea in the ovaries. The case was stated as follows: "The breaking of the Graafian follicles occurs during the œstrus as a result of congestion which began in the theca folliculi at about the same time as the congestion of the stroma of the uterus and vagina. And finally when the regenerative growth of the uterine mucosa sets in, the ovaries then possess new corpora lutea in an active state of differentiation which have been derived from these recently ruptured follicles." The presence of the new active corpora lutea suppresses the final steps in the development of the almost mature Graafian follicles in both ovaries, whether the corpora lutea be located in only one of the ovaries or both. When the corpora lutea become less active and their degeneration has proceeded to a certain extent, another ovulation may then take place. Therefore, the functions of the corpora lutea are probably, first, by their presence and activity to inhibit ovulation or to determine its time, and, secondly, to preserve the structure of the uterine wall and prevent its degeneration.

Loeb has attacked the problems of corpora lutea function in the guinea-pig in a more direct experimental way than have other investigators. Yet while studying the effects of corpora lutea removal on the length of the ovulation period, he has been handicapped by the fact that his animals, after the initial operation, were later killed for examination and thus were no longer available for a continuation of the experiment. Only one observation was obtained from any particular female. The

effects on the lengths of the ovulation periods of the removal of corpora lutea or the application of its extracts could be investigated to great advantage on guinea-pigs in which the œstrous cycles are definitely known and followed through a number of consecutive periods. This could readily be done by the method before described. This method is also of value in locating the early stage of developing eggs and in making exact matings for studies on fertilization, etc.

Attention may be called to further slight objections that might be raised in considering Loeb's last paper. He studies the conditions in the structure of the uterine wall removed from females that had copulated shortly before, as well as, uteri from uncopulated females, and states, page three: "The sperm fluid present in the lumen of the uterus exerts a pressure on the surface epithelium and may thus contribute to the harmful influence of the leucocytes." This idea is incorrect since it may be clearly shown that the action of the leucocytes is equally as harmful in the destruction of the uterine epithelium during the œstrous period of virgin females.

In a similar connection Loeb also finds, page 11, that the number of leucocytes in the uterine mucosa is much smaller in animals that have not copulated. Again, page 16, "A few leucocytes can also be seen in the uterus of animals in which copulation had been prevented. . . . In such cases (non-copulated animals) also some degenerative changes occur in the uterine epithelium, but they are less marked than in animals which had copulated." These statements are not entirely in accord with our findings since there is no such marked discrepancy between virgin females and those that have copulated. Such conclusions are probably due to the fact that the uteri examined were not removed from the non-copulated females at the maximum moment of leucocyte migration and degeneration of the uterine wall (our "third stage"). Loeb had no exact means of knowing the comparable stages in copulated and non-copulated females.

The uterine mucosa of our virgin females may show leucocytes to be equally as abundant at comparable stages as the uteri from specimens after copulation. It must be recognized in this con-

nection that each stage in the condition of the uterine wall during the œstrous is of short duration and unless the uterus be removed at a given time, the abundance and position of the leucocytes and the condition of the uterine wall will be changed. Chance was against Loeb's removing the uteri from the non-copulated females at the moment of maximum leucocyte migration, since he had no exact means of knowing when this would occur without having first observed copulation.

Active migration and accumulation of leucocytes may be observed in the entire absence of sperm fluid. The rôle of this fluid and the modification of the shedding or sluffing off of the vaginal and uterine epithelium in its presence was fully brought out in the discussion above of the vaginal plug.

Loeb's estimation of the ovulation times and uterine changes from microscopic examination of fixed specimens does not make it possible to know within a few hours, or even days, of the exact moment of ovulation in a given living individual. He states, however, on page 31, that to determine the effects of the removal of the corpora lutea on the duration of the sexual cycle, it "was necessary to determine the length of the cycle in the normal guinea-pig." Not only is this necessary, particularly in view of the wide variations Loeb finds in the normal sexual cycle among different individuals, but it is better or even necessary, to know the actual length and variations of the sexual cycle in the given specimen experimented upon. As evidence of the correctness of the last statement, we may cite Loeb's method and results in determining the normal cycle lengths. This was done "by observing the time of heat of a guinea-pig and by examining the uterus and ovaries at known intervals" (after removal from different individuals). Such examinations were made on many specimens that had to be either killed or operated upon. The following ovulation intervals were thought to be the normal sexual cycles, page 31, "We found the length of the sexual period to be usually sixteen to eighteen or nineteen days; sometimes the new ovulation may take place as early as fifteen days after copulation. In two exceptional cases we observed the new ovulation as early as thirteen and a half to fourteen and a half days." The sexual cycle, therefore, varies in length from

thirteen and a half to nineteen days, a range of almost six days.

On this basis it is seen to be practically impossible to state within a day or so of when the next ovulation will take place in a female that has just passed the "heat period." And the "heat period" was very indefinitely known unless copulation had been observed. Thus, as a usual practice, in order to prevent pregnancy following a copulation used to prove the existence of heat, the oviducts were previously tied. Such a procedure might easily modify to some degree the sexual cycle and is inconvenient for further study of the animal.

Any significant experimental modification of the ovulation intervals could be readily detected by a simple examination of the microscopic structure of the vaginal fluids collected from a guinea-pig.

Finally, the "signs of heat" recorded from a report by Miss Lathrop, an animal breeder, are, according to our experience in examining and mating guinea-pigs, generally inaccurate and of little value for use in experimental studies.

5. Summary.

1. The œstrous cycle in the guinea-pig is very definitely limited in length. Ovulations follow one another every fifteen to seventeen days in younger individuals, while in old females the period is slightly lengthened, in exceptional cases to eighteen days.

2. These individual variations are readily controlled by the method of vaginal examination described in this and a former article, so that the actual moment of ovulation in any given female may be determined to within almost an hour of the rupture of the Graafian follicles.

3. During the period of sexual inactivity, the dioestrum, as well as during pregnancy, the orifice of the vagina is completely closed by an overgrowth of epithelium which we have termed the "vaginal closure membrane." This membrane ruptures just before or during the first stage of the œstrus in non-pregnant females and before parturition in the pregnant. It always reforms to close the vagina shortly after the "heat period" has

passed. The presence of this closure membrane is therefore positive evidence that the time of a new ovulation has not been reached. When the membrane is ruptured and the vagina open, the ovarian condition may then be determined by examination of the fluid content of the vagina as described. A knowledge of this closure membrane greatly facilitates the examination of females in locating the beginning of the œstrus.

4. Copulation takes place in the guinea-pig during stage one of the œstrous period and ovulation occurs, as we previously showed, at the end of the second stage or the beginning of the third. Long ('19) finds exactly the same to be true for the rat.

5. At the time copulation occurs, the vagina is filled with a clear foamy mucus, and this is the only time at which no leucocytes are present in the fluid. Both the nature of the vaginal fluid at this time and the absence of leucocytes contribute to the success of copulation and fertilization.

6. A vaginal plug is formed a few minutes after copulation, during the first stage, and remains in the vagina for only a short time, being expelled during the fourth stage of the œstrus.

The core or center of the vaginal plug is composed chiefly of coagulated seminal fluid. This is enclosed within an envelope of epithelial cells, being simply the sluffed-off mucosa from the wall of the uterus and vagina. This epithelial cover which is thrown off at every œstrous period is loosened or dissolved away from the uterine and vaginal wall by an enormous invasion of leucocytes which progresses from the first to the third stage. The breaking away from the vaginal wall of this enveloping epithelium causes the plug to fall out of the vagina during the fourth stage. After the expulsion of the vaginal plug, the mouth of the vagina is closed by the growth of the vaginal closure membrane.

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THE INDIVIDUALITY OF THE GERM-NUCLEI DURING THE CLEAVAGE OF THE EGG OF CRYPTOBRANCHUS ALLEGHENIENSIS.

BERTRAM G. SMITH.

Early observers of the process of fertilization described the meeting of the sperm-nucleus and the egg-nucleus, and their complete fusion to form a single zygotic nucleus, the primary nucleus of the embryo. Later it was found that in many cases, though apparently not in all, the two germ-nuclei merely become apposed without fusion. In tracing their further history it was shown, in certain cases, that the germ-nuclei give rise to two independent groups of chromosomes which separately enter the equatorial plate and whose descendants pass separately into the daughter-nuclei. "Later observations have given the strongest reasons for believing that, as far as the chromatin is concerned, a true fusion of the nuclei never takes place during fertilization, and that paternal and maternal chromatin *may* remain separate and distinct in the later stages of development—possibly throughout life" (Wilson, 1900, p. 204). At the present time there is much to warrant the belief that a fusion of maternal and paternal chromatin never takes place in the somatic cells, while in the lineage of germ cells nothing approaching fusion occurs until a pairing of maternal and paternal chromosomes, called synapsis, takes place in preparation for the maturation divisions; then for the first time maternal and paternal chromosomes are brought together in intimate and orderly union, in some cases amounting to fusion.

This conclusion, which is of the most fundamental importance since it vitally concerns the mechanism of heredity, is based largely on indirect evidence; for in almost every case apparently insuperable obstacles have been encountered in the attempt to trace the respective maternal and paternal chromatin-complexes through the resting stage of the nuclear cycle. In only a very

few cases has the individuality of the germ-nuclei during this stage been demonstrated with any considerable success; the observations of Häcker (1892), Rückert (1895) and Conklin (1901) are of most importance in this connection and will be discussed later.

In the developing egg of the amphibian *Cryptobranchus allegheniensis* I have found material very favorable for the study of this problem. The early cleavage nuclei are invariably double throughout the entire resting stage, each consisting of two separate though closely apposed nuclear vesicles, and the origin of each vesicle from a single germ-nucleus has been clearly traced. Indeed, my observations indicate that typically the germ-nuclei maintain their individuality throughout cleavage, though certain irregularities, real or apparent, occur with increasing frequency in the later segmentation stages.

Technique.

Most of the sections used in making the observations recorded in the present paper were cut from eggs killed with bichromate-acetic-formalin. This mixture, made up in the right proportions (Smith, 1912, I.), preserves the form of the egg perfectly in all stages, enables it to be sectioned by the paraffin method, and gives very satisfactory histological and cytological results. For comparison, sections were made of a few eggs killed in Zenker's fluid, also in corrosive-acetic-formalin. These mixtures, particularly Zenker's fluid, gave excellent results in the fertilization and early cleavage stages, but in the blastula and gastrula stages their use is not advisable since the roof of the blastocœle or gastrocœle collapses during the process of infiltration with paraffin.

Most of the observations were made on sections of eggs stained *in toto* with Grenacher's borax-carmine, differentiated in acid alcohol, and counterstained on the slide with a mixture of Lyons blue and picric acid. This treatment leaves the chromatin stained red, the yolk granules green, the centrospheres and cytoplasmic structures blue. The method has the advantage of rapidity of manipulation, an important consideration since a

large number of slides are required. For the main purposes of this investigation the differentiation obtained by this method, used with care, is entirely adequate, but as a precaution against possible inaccuracy a parallel series was stained with Heidenhain's iron hæmatoxylin, which as might be expected gave better preparations, and is especially useful for the study of chromosomal vesicles.

All the figures excepting those of Plate IX. were outlined by means of a camera lucida and drawn with a magnification of about 600 diameters; in the process of reproduction they were reduced one third, and thus appear with a magnification of about 400 diameters. In all drawings of horizontal sections the orientation with reference to the plane of first cleavage is the same, and conforms to the diagrams in Plate IX.

In most cases the double nature of the cleavage nuclei appears to best advantage in horizontal sections, and might readily be overlooked were observations confined to vertical sections. It seems likely that most investigators studying the amphibian egg have confined their observations to vertical sections, since these give a better idea of the gross structure; and this circumstance leads us to suspect that conditions such as are here described for *Cryptobranchus* may occur in other amphibians and need only to be revealed by the study of horizontal sections.

Observations.

The early history of the fertilization stage of *Cryptobranchus allegheniensis* has been given in a previous paper (Smith, 1912, I.). The superficial cleavage pattern has been described in another contribution (Smith, 1912, II.).

At the time of the meeting of the germ-nuclei it is usually impossible to tell with certainty which is the egg-nucleus and which is the sperm-nucleus, but this circumstance does not affect the validity of the results recorded in this paper. The germ-nuclei do not fuse, but come to lie side by side with nuclear membranes intact (Figs. 1–4). During the long resting stage that precedes the formation of the first cleavage spindle, each germ-nucleus maintains strict individuality: there is close asso-

ciation, but no actual union, and certainly no mingling of structural contents. Hence, although it is convenient to employ such terms as "union of the germ-nuclei," and to speak of the "cleavage-nucleus" or "segmentation-nucleus" as if it represented a single entity, it should be understood that such expressions are loosely used, and the word "fusion" in this particular case is altogether inapplicable. It is the purpose of this paper to show that this unity or individuality of the respective germ-nuclei is not confined to the fertilization stage, but persists with great regularity during early cleavage, and with some apparent irregularities throughout the later cleavage and even into the gastrula stage.

The length of time intervening between the meeting of the germ-nuclei and the rupture of their nuclear membranes during the formation of the first cleavage spindle must be considerable, for resting germ-nuclei have been observed in eggs varying in age from twelve to twenty-three hours after fertilization. The precise time of formation of the first cleavage spindle has been determined in only one egg, which was killed twenty-six hours after artificial fertilization.

Since the germ-nuclei usually come to lie in the same horizontal plane, the most fruitful observations are obtained through the study of horizontal sections. The two asters, formed by the division of the single aster accompanying the sperm-nucleus on its journey to meet the egg-nucleus, take up positions on opposite sides of the resting germ-nuclei, in the same horizontal plane, and close to the region of contact of the germ-nuclei (Figs. 3 and 4). The initiation of the process of mitosis as evidenced by the appearance of distinct chromosomes and the rupture of the nuclear membranes does not take place in the two germ-nuclei simultaneously; one of the germ-nuclei becomes active somewhat in advance of the other (Figs. 5 and 6), thus furnishing evidence of some degree of physiological as well as structural independence.

In the fully-formed mitotic figure the two groups of chromosomes, of maternal and paternal origin respectively, remain visibly distinct (Fig. 7). This has been determined beyond a

doubt in a number of cases, with no conflicting evidence. In the anaphase each of the original chromosome groups has divided equally (through the splitting of individual chromosomes), the two halves going to different poles of the spindle. Each daughter-nucleus thus receives two distinct groups of chromosomes, derived from the egg-nucleus and the sperm-nucleus respectively. Although in the anaphase and early telophase (Fig. 8) the paired groups of chromosomes are not always very sharply separated, yet there is no mingling of maternal and paternal chromosomes. Finally, in the newly-formed daughter-nucleus each parental group of chromosomes becomes metamorphosed into a separate nuclear vesicle (Figs. 9 and 10). Except for a diminution in size, which is afterwards partially made good by growth, each daughter-nucleus closely simulates the original cleavage-nucleus with its two resting germ-nuclei.

The precise stage with newly-formed vesicular daughter-nuclei has not been found in horizontal sections, hence all the figures of this stage are taken from vertical sections (Figs. 9–12). During the late phases of mitosis the two daughter-nuclei move some distance apart; their path of separation is marked by a broad trail of cytoplasm sharply defined from the surrounding yolk. The division of the centrosphere or "attraction sphere" takes place shortly before the daughter-nucleus assumes the vesicular condition. The first cleavage furrow is not yet formed, but its position, in a plane at right angles to the path of separation of the two daughter-nuclei and midway between them, is visibly indicated by a condensation of cytoplasm in the blastodisc.

Aside from the fact that the two nuclear vesicles of a newly-formed daughter-nucleus are always situated approximately equidistant from the plane of first cleavage, the orientation of these nuclear vesicles with reference to each other is decidedly variable. Since the two original germ-nuclei at the time of meeting usually lie in the same horizontal plane (Figs. 2–4), we should expect the same thing of the two vesicles of a daughter-nucleus; but in the preparations studied these were more often found to lie in a plane oblique to the horizontal, and sometimes one vesicle lies directly above the other (Figs. 9 and 10). It should be remembered,

however, that the original germ-nuclei do not always lie in the same horizontal plane (Fig. 1). Moreover, the stage with newly-formed daughter-nuclei was not represented in horizontal sections, which are naturally most favorable for the discovery of nuclei consisting of vesicles lying in the same horizontal plane. There is no external landmark by means of which this stage may be recognized, and it is passed through very rapidly; in the making of horizontal sections the available material was exhausted without finding the precise stage desired.

In the study of the subsequent behavior of the nuclei the early cleavage furrows serve as convenient landmarks for orientation, and it is necessary to keep in mind the elementary fact that the second cleavage furrow forms at right angles to the first, and that a similar alternation in the direction of successive cleavage furrows is characteristic of the entire process of cleavage. Of more immediate importance to us is the correlated fact that the second nuclear division occurs in a direction at right angles to the first nuclear division, and that this alternation in the direction of successive nuclear divisions is repeated throughout cleavage. To be sure, this is strictly true only in the early cleavage stages, for there is increasing irregularity in the direction of both cleavage furrows and nuclear divisions throughout the later stages of cleavage. Incidentally, it should be noted that nuclear division always precedes the formation of the corresponding cleavage furrow.

In preparation for the second nuclear division, the two asters of a single daughter-nucleus take up positions on opposite sides of the nucleus, in the same horizontal plane and with a line connecting them parallel to the first cleavage furrow which is now usually in process of formation (Figs. 12–20; 23–26). In case the two nuclear vesicles of a single daughter-nucleus are not already ranged midway between the two asters, they rotate to this position (compare Figs. 15–16 with Figs. 17–18). Typically, the two nuclear vesicles come to lie in the same horizontal plane (Figs. 17–20, 23–26); but this is not the invariable position, for in three different eggs they were found to lie one above the other, as exemplified in Figs. 21 and 22. In any case the final

position assumed insures an equal division of maternal and paternal chromatin-groups in the ensuing mitosis. Thus the second cleavage mitosis (Figs. 27 and 28) is in all essential respects a repetition of the first, though the direction of the spindle is different. Throughout the entire segmentation period there is nothing in the behavior of the nuclei more impressive than the precision and regularity with which the two nuclear vesicles of a single nucleus are ranged side by side in the plane of the equatorial plate in preparation for the formation of the spindle.

In the resting stage following the second mitosis the two nuclear vesicles of each nucleus usually lie in the same horizontal plane (Figs. 29, 30, 32); their entire orientation indicates their derivation from the two groups of chromosomes at one pole of the spindle in the anaphase and early telophase of the preceding mitosis (Fig. 28). Occasionally, though not so often as in the preceding resting stage, one nuclear vesicle is found situated above the other (Fig. 31).

In *Cryptobranchus* the third cleavage furrows are vertical and intersect the second furrows at some distance from the first (Plate IX., Fig. 45, *J*). Thus the third cleavage furrows are nearly parallel to the first, but diverge slightly from it.

In preparation for the third cleavage mitosis the two asters take up positions on opposite sides of the nucleus, in such a situation that a line connecting them is nearly parallel to the plane of second cleavage (Figs. 33, 34; Plate IX., Fig. 45, *H*). In all cases the two nuclear vesicles entering the mitotic figure are ranged equidistant from the two asters (Figs. 33, 34, 35), and in a slightly earlier stage may be found rotating to this position (Fig. 32). Thus in the third cleavage mitosis (Fig. 36) the segregation and equal division of maternal and paternal chromosome groups is maintained. The direction of the axis of the spindle is at right angles to the preceding mitosis, and parallel to the first (Plate IX., Fig. 45, *H* and *I*). Upon the completion of mitosis the third cleavage furrows form as described in the preceding paragraph. At this time the four nuclei on the same side of the second cleavage furrow sometimes lie precisely in

the same vertical plane, so that they are all visible in a single section taken parallel to the second cleavage furrow.

The fourth cleavage furrows combine to form a circular, or more often oval, figure with its longer axis coinciding with the plane of second cleavage. This circular cleavage divides the egg very unequally, cutting off eight micromeres from eight comparatively large macromeres. Preparation for this cleavage involves rotation of the two nuclear vesicles of a single daughter-nucleus of the third cell division from their original position as shown in Fig. 37 to the position shown in Fig. 38. This latter orientation insures the maintenance of the segregation of the germ-nuclei in the fourth mitosis (Figs. 39–42). As in the previous divisions, the maternal and paternal chromosome groups have here been traced through the various phases of mitosis to the late anaphase and early telophase, where they are still distinctly separate. The position of the vesicles of the newly-formed daughter-nucleus (Figs. 43 and 44) indicates that one is of maternal origin, the other paternal. The results as regards the orientation of the nuclei up to this point are summarized in the diagrams of Plate IX., Fig. 45.

In the later cleavage divisions there is increasing irregularity in the direction of spindle axes and cleavage furrows, but this does not constitute any serious obstacle to tracing the history of the germ-nuclei since the really important point is the orientation of the nuclear vesicles with respect to the axis of the spindle. As already indicated, this is invariably such as to insure the segregation and equal representation of maternal and paternal chromosome groups in the daughter-nuclei. As the nuclei become smaller, it sometimes is difficult to distinguish the two groups of chromosomes in the late phases of mitosis, but comparison with slightly earlier and later phases leaves no doubt as to the continuity of the respective germ-nuclei. Until an advanced cleavage stage, the double structure of the resting nuclei is almost always clearly demonstrable. As the nuclei become more numerous, it becomes easier to find nuclei cut in such a manner as to show distinctly the double structure. In the more active regions of the egg, the synchronism of division

of the different cells is maintained to the late blastula stage, so that there is little likelihood of confusing cell-generations. Between micromeres and macromeres there is a fine gradation in phases of the nuclear cycle, so that a close series may be readily obtained. The individuality of the germ-nuclei has been traced without a break to an advanced cleavage stage, and it would not be difficult to illustrate this entire result with an adequate series of figures, but such a procedure would require an enormous amount of time and unduly extend the limits of this paper.

Beginning with about the seventh cell generation certain irregularities, real or apparent, occur with increasing frequency to mar the mechanical precision of the events thus far described in the history of the germ-nuclei. In the resting stage the two nuclear vesicles of a single nucleus are often deeply lobed, sometimes in such a manner as to give the impression that the nucleus is made up of many vesicles. In the late telophases the nucleus is often really made up of several or many separate though closely aggregated vesicles, but this is undoubtedly a part of the usual process of mitosis, as a more searching examination of these late phases throughout cleavage will readily show.

The transformation of the daughter-nucleus into the vesicular condition must take place with considerable rapidity, especially in the early cleavage stages, for some phases of this process have not been observed earlier than the fourth mitosis, and only occasionally in the fourth and fifth mitoses. In more advanced cleavage there is little difficulty in finding material representing the entire history of the telophase.

In the early anaphase, the long thread-like chromosomes are V-shaped, with the limbs of the V almost parallel and very straight; the apex is directed away from the equatorial plate. In a slightly later phase, which we may call the late anaphase, the chromosomes are still roughly V-shaped but finely undulating except at their free ends. A little later (early telophase) each chromosome becomes much convoluted, often coiled in a loose irregular spiral; this change begins at the apex and proceeds toward the free ends. The next step consists of the metamorphosis of each chromosome into a chromosomal vesicle, a

process which I have been able to follow in considerable detail, but will not attempt to describe here. About this time the centrosphere or "attraction sphere" divides.

During the anaphase and early telophase the maternal and paternal chromosome groups are almost always clearly separated, sometimes with a broad space between the two groups. The segregation of the chromosomal vesicles into two groups, maternal and paternal respectively, is usually evident. In the late telophase the chromosomal vesicles of each group unite to form separate nuclear vesicles which for a time show indications of their manner of origin through the persistence of lobes separated by deep clefts. The inner boundaries of the chromosomal vesicles persist for a time as partitions in the nuclear vesicles, but finally disappear wholly or in part. In the late blastula the vesicular nucleus in many cases retains a lobed structure throughout the resting stage. It is possible that in some cases the fusion of chromosomal vesicles to form vesicular nuclei of the usual duplex type is never completed, for in the late blastula and early gastrula nuclei consisting of three, four or even more well-rounded vesicles are not uncommon.

How much longer and to what extent during the development of the embryo a strict separation of the germ-nuclei is maintained I have not attempted to determine, but it is not necessary to conclude that it continues throughout the life of the organism, either in the somatic or the germ cells. In the discussion it will be shown that the individuality of the germ-nuclei is not incompatible with a mingling of maternal and paternal chromosomes, and there is no biological necessity for a strict separation of the germ-nuclei in any stage from fertilization to synapsis.

Discussion.

In 1875 Oscar Hertwig and Hermann Fol showed that the fertilized egg contains two nuclei, one belonging to the egg itself and the other introduced by the spermatozoön. While the earliest observers of the process of fertilization, notably Auerbach, Strasburger and Hertwig, described the complete fusion of these germ-nuclei to form the first embryonic nucleus, called by

Hertwig the cleavage-nucleus or segmentation-nucleus, later observations showed that such is not always the case. In 1881 Mark demonstrated that in the slug *Limax* the two germ-nuclei do not fuse; after coming together they persist during the formation of the two cleavage centers, then their membranes gradually disappear. Two years later Van Beneden showed that in *Ascaris*, not only do the germ-nuclei become apposed without fusion, but each gives rise to an independent group of chromosomes which contribute equally to the formation of the daughter-nuclei. Thus the foundation of the doctrine of the biparental character of the nuclei of sexually-produced organisms was laid down by Van Beneden. In many animals, and in some plants (gymnosperms), the independence of the formation of the maternal and the paternal chromosome groups following fertilization has been established by direct observation, though the demonstration has seldom been carried beyond the first cleavage stage. On the other hand there are some animals, and many plants, in which the germ-nuclei meet and fuse while in the resting condition, so that in the chromatin of the resulting nucleus maternal and paternal contributions cannot be readily distinguished.

Häcker (1892 and 1895) and Rückert (1895) found that the germ-nuclei of *Cyclops* do not fuse but preserve their individuality throughout at least a considerable period of the cleavage of the egg. In mitosis the two groups of chromosomes, of maternal and paternal origin respectively, remain distinct and bilaterally distributed, while each resultant daughter-nucleus in the resting stage consists of two closely apposed but structurally separate vesicles.

In 1901 Conklin described the double structure of the cleavage nuclei of *Crepidula* in certain stages of the nuclear cycle, and pointed out that the two halves which at times appear as distinct entities are almost certainly to be regarded as of maternal and paternal origin respectively. "This separateness is most easily observed in the telophase of each division, though in some cleavage cells it may be seen in the prophase also, or even throughout the resting period. At the time when the daughter-nuclei are being formed the chromosomal vesicles fuse into two groups

which are closely pressed together but still separated by a partition wall, as Rückert has shown to be the case in *Cyclops*. Gradually this partition wall disappears, being preserved longest on that side of the nucleus nearest the centrosome. Here a groove is formed on one side of the nucleus which marks the line of contact between the two halves. In some cleavage cells this groove is visible throughout most of the resting period; in others it disappears during the greater part of the resting period, though it may reappear in the following prophase; in all cases, however, the partition wall and groove reappear in the next succeeding telophase, when it is again formed in the manner described above. I have observed the double character of the nucleus in the telophase of every cleavage up to the 29-cell stage, and in several of the later cleavages up to the 60-cell stage, though it becomes more difficult to see as the nuclei grow smaller. . . . It still remains to show that these double nuclei really represent the egg and sperm nuclei which have not yet lost their individuality. This cannot be demonstrated in *Crepidula*, for the reason that this double character is not apparent at every stage in the nuclear cycle, but it is extremely probable" (Conklin, 1901). Additional observations recorded by Conklin in support of his interpretation may be summarized as follows: (1) In the metaphase of the first cleavage division the maternal and paternal germ-nuclei are represented by separate groups of chromosomes; in the early anaphase these groups of chromosomes can no longer be distinguished, but the nuclei are clearly double in the immediately following late anaphase and telophase, and the position of the partition wall in these double nuclei corresponds to the plane of contact between the germ-nuclei. (2) The groove which is found on one side of the nucleus in the telophase of the first cleavage mitosis persists well into the resting stage, and a corresponding groove is found in the same position in the prophase of the second mitosis. The central spindle for the second cleavage mitosis lies in this groove, and thus the amphiaster actually lies in the only plane in which it would be possible to halve the two parts of the double nuclei. Although cleavage divisions successively alternate in direction, unequal division of the double nuclei is

prevented either by a rotation of the nucleus during the resting stage, or by a rotation of the spindle in the early stage of mitosis. (3) In certain abnormal cleavages the double nuclei are really two entirely separate nuclei lying side by side within a single cell. (4) In each of the germ-nuclei, before they come into contact, there is a single nucleolus; these nucleoli disappear in the prophase of the first cleavage, but in the succeeding telophase a single nucleolus generally appears in each half of each daughter-nucleus. The same is true of the succeeding cleavages, so that each nucleus throughout the cleavage usually has two nucleoli in the telophase or early resting stage.

Beard (1902) described a double structure of the resting stages of the nuclei of the early germ cells of *Raja batis;* these were not traced earlier than a late gastrula stage, but influenced by the findings of Häcker and Rückert, Beard did not hesitate to interpret the double nuclei as consisting of distinct maternal and paternal halves.

Jenkinson (1904) gives some interesting figures of the fertilization and first cleavage stages of *Axolotl.* The germ nuclei meet without fusion, and the chromosomes appear separately in each pronucleus while the nuclear membranes are still intact. In some cases at least, these two chromosome groups remain distinct in the equatorial plate after the dissolution of the nuclear membranes. Scant attention is paid to these features in the text of Jenkinson's paper, which is concerned with other matters, but the author states that he has found two distinct sets of chromosomes in some preparations of the fertilization spindle of *Triton.*

In 1904 Moenckhaus described the independence of the maternal and paternal chromosome groups in the early cleavage spindles of the hybrids produced by fertilizing the eggs of *Fundulus* with *Menidia* sperm. The difference in the size and shape of the chromosomes of the two species makes the identification of the maternal and paternal chromosomes in the case described a matter of certainty.

Pinney (1918) found that two nucleoli are typically present in the nuclei of normal *Ctenolabrus* blastoderms, and cited evi-

dence supporting the view that in these double nucleoli observed in fishes we are dealing with parental homologues.

Concerning *Cryptobranchus allegheniensis*, the writer believes that the observations recorded in the present paper establish beyond question the complete separation of maternal and paternal germ-nuclei to a late blastula stage at least. The separation is particularly marked during the resting stage of the nuclear cycle, precisely where most investigators working with other species have encountered the greatest difficulty.

The observations thus far cited indicate that in certain forms the individuality of the germ-nuclei during early embryonic development is maintained by complete separation of the nuclear material derived from the egg and the spermatozoön respectively. It has already been indicated that this segregation is not by any means a universal phenomenon. Is it possible that the individuality of the germ-nuclei may be maintained, in all essential respects, in those other cases where there is a mingling of chromatin derived from the two germ-nuclei? Let us first examine the facts that require explanation.

In all cases where the germ-nuclei fuse into a single vesicular nucleus before the formation of the first cleavage spindle, mingling of maternal and paternal chromosomes may be expected. Sax (1918) has recently described two cases in flowering plants, *Fritillaria* and *Triticum*. In *Fritillaria* the germ-nuclei usually unite while in the resting condition, although occasionally they are in the spireme stage at the time of fusion. The presence of a single spireme in the zygote could not be demonstrated. In *Triticum* the sperm-nucleus is small and almost homogeneous in structure even while in contact with the egg nucleus. The sperm-nucleus enters the egg-nucleus and there forms a separate compact spireme; at the same time the spireme of the egg-nucleus is formed. In both *Fritillaria* and *Triticum* the maternal and paternal chromosomes are formed independently, but they are not found in separate groups.

Moenckhaus (1904) found that after the first few cleavage divisions in his hybrid teleost eggs the chromosomes of maternal and paternal origin mingled indiscriminately upon the equatorial

plate, and the observations of Morris (1914) are in agreement.

Metz (1916) has found in the diptera a pairing of homologous chromosomes, simulating synapsis, which occurs in all tissues, somatic as well as germinal; this association of maternal with paternal chromosomes was found in late cleavage and during all later stages of embryonic development. From the following statement by Overton (1909) we have a suggestion of a similar occurrence in plants, though attention is directed more particularly to the matter of genetic continuity of individual chromosomes: "In the somatic nuclei (of certain plants) the chromosomes are represented during rest by definite visible bodies, the pro-chromosomes, which are arranged in parallel pairs, with apparent linin intervals. These heterogeneous spirems, the homologous portions of which have early become associated in pairs, probably remain distinct throughout the life-history of the sporophyte."

In spite of the mingling and even paired association of maternal and paternal chromosomes, there are reasons for believing that the two kinds of chromosomes maintain their independence until gametogenesis. This leads us to a consideration of the doctrine of the genetic continuity of individual chromosomes, which goes further than the principle of duality of the embryonic nuclei, but confirms it as a universal law.

The remarkable constancy in the number of chromosomes throughout the cells of a given organism and species has long been known, and affords important evidence for the view that the chromosomes are persistent as individual structures. To be sure, it sometimes happens in mitosis that one or more chromosomes belonging to one daughter-group, accidentally become included with the other group so that one of the daughter-nuclei has fewer, the other more, than the normal somatic number; but such an occurrence is very exceptional, and in subsequent divisions of these cells the number of chromosomes appearing is not the normal, but the increased or diminished number (Boveri, 1890). Whatever the number of chromosomes entering into the formation of a resting nucleus, the same number afterwards issues from it.

In 1883 Van Beneden showed that in *Ascaris* the spermatozoön brings in just as many chromosomes as are contained in the egg. As a result of a careful study of mitosis in epithelial cells of the salamander, Rabl (1885) concluded that the chromosomes do not lose their individuality at the close of division, but persist in the chromatic reticulum of the resting nucleus. Boveri (1887 and 1888) supported Rabl's hypothesis on the ground of his own studies and those of Van Beneden on the early stages of *Ascaris*. Boveri demonstrated in *Ascaris* that in the formation of the spireme the chromosomes reappear in the same position as those which entered into the formation of the reticulum, precisely as Rabl had maintained. As the long chromosomes diverge, their free ends are always turned toward the equator of the spindle, and upon the reconstruction of the daughter-nuclei these ends give rise to corresponding lobes of the nucleus, which persist throughout the resting stage. At the succeeding division the chromosomes reappear exactly in the same position, their ends lying in the nuclear lobes as before. These observations were afterwards confirmed by Herla (1893), and more recently Sutton (1902) has observed practically the same thing in *Brachystola magna*. Boveri (1891) concluded that the chromosomes must be regarded as individuals that have an independent existence in the cell, and expressed his belief that "we may identify every chromatic element arising from a resting nucleus with a definite element that entered into the formation of that nucleus, from which the remarkable conclusion follows that in all cells derived in the regular course of division from the fertilized egg, one half of the chromosomes are of strictly paternal origin, the other half of maternal."

Herla (1893) and Zoja (1895) have shown that if in *Ascaris megalocephala* the egg of variety *bivalens*, having two chromosomes, be fertilized with the spermatozoön of variety *univalens*, having one chromosome, the three chromosomes reappear at each cleavage, at least as far as the twelve-cell stage; and according to Zoja, the paternal chromosome is distinguishable from the two maternal at each step by its smaller size. "We have thus what must be reckoned as more than a possibility, that every cell

of the body of the child may receive from each parent not only half of its chromatin substance, but one half of its chromosomes, as distinct and individual descendants of those of its parents" (Wilson, 1900).

Boveri (1909) found in *Ascaris* that in sister cells preparing for division the configuration of the groups of chromosomes is the same. The similarity of the sister cells is explicable on the view that the chromosomes retain during the resting stage the same shape and size and relative location that they had at the end of the preceding division. In cells of these same embryos that are not sister cells, a great variety of arrangements of the chromosomes is found, and no two arrangements are so nearly alike as are those found in sister cells.

Other evidence for the continuity of individual chromosomes is derived from those cases where the reconstruction of the resting nucleus takes place through the metamorphosis of each chromosome into a hollow vesicle, and the aggregation or fusion of these chromosomal vesicles to form a single nucleus. Such, to be sure, is not the only type of telophase (Wilson, 1900, p. 71), but it takes place in many segmenting ova and in some spermatogonia. According to Conklin (1902), in the late stages of mitosis of the segmenting egg of *Crepidula* the chromosomes enlarge to form vesicles and these unite into a resting nucleus; the nuclear membrane is composed of the outer walls of the vesicles, while the inner walls stretch through the nucleus as chromatic partitions; the chromosomal vesicles from the egg and sperm nuclei respectively remain distinct longer than those from the same germ nucleus. Vesicular chromosomes have been described in fish eggs by Moenckhaus; the individual vesicles fuse with their neighbors and these larger ones with each other until at last the entire nucleus is simply one great vesicle, which is at first lobed, but later is well rounded. Wenrich (1916) says of *Phrynotettix:* "The spermatogonial divisions showed that each chromosome forms a sac or vesicle in the earlier telophases, and that it expands and becomes diffused within these vesicles; that, although the vesicles appeared to coalesce, there is always a remnant of each chromosome visible in the center of the region occupied by the

vesicle, and that in the prophase the chromatin concentrates about this remnant or core and there forms a 'spirally coiled thread, which develops into a prophase chromosome." Richards (1917) has shown that in the cleavage and gastrula stages of *Fundulus* and *Coregonus* the chromosomes in the telophase of mitosis enlarge to form vesicles which remain distinct though compactly massed together during the resting stage. The new chromosome arises within a vesicle, through the aggregation of its granules; thus there is genetic continuity of individual chromosomes. According to Richards, the polarity of the cell is manifested in the arrangement of the elongated vesicles. When the centrosome divides, the cell acquires a new axis at right angles to the old, in a line connecting the two asters; the chromosomes, when formed, orient themselves with respect to the new axis. Pinney (1918) figures chromosomal vesicles in the telophase of dividing blastomeres of *Ctenolabrus* × *Fundulus* hybrids. Some observations on chromosomal vesicles in *Cryptobranchus allegheniensis* are included in the present paper. I have examined a few sections of *Coregonus* blastoderms belonging to the collection of the Michigan State Normal College and the material appears to be exceptionally favorable for the study of chromosomal vesicles.

Thus it has been proved that in the fertilized egg one half of the chromosomes are derived from the father and one half from the mother, and that at every division of the egg the chromosomes also divide in such a manner that their progeny are distributed in equal number to all the cells of the egg. Further, there is genetic continuity between each individual chromosome that enters the resting stage and a corresponding chromosome that emerges in preparation for the next division. The conclusion is thus reached that the fertilized egg, and all the cells derived from it, contain a double set of chromosomes, paternal and maternal. This conclusion implies that at every step the respective chromosome groups preserve their independence, however much they are mingled with one another.

This conclusion is further strengthened by observations of individual differences, both morphological and physiological,

between the chromosomes of a given simplex group, and by the behavior of the chromosomes in synapsis. The chromosomes of a single group, maternal or paternal, are not precisely alike, but differ among themselves in size, form and genetic potency (Montgomery, 1901; Sutton, 1902 and 1903; Morgan, 1915). In certain species the size differences are very marked, so that the chromosomes of a single germ-nucleus may be arranged in a graded series; these size differences are constant from one cell-generation to the next, so that individual chromosomes may be identified in successive cell-divisions. In other species where the visible differences are not so marked we have evidence that physiological differences exist, for Boveri (1907) has shown the strong probability that normal development of the egg is possible only in the presence of at least a single set of qualitatively different chromosomes. Thus the concept of the individuality of the chromosomes has been extended to include not merely the genetic continuity of each particular chromosome, but also its idiosyncrasy or specificity.

In any biparental organism, the duplex chromosome group is composed of two equivalent parental series or simplex groups, in which each individual chromosome is homologous with a very similar chromosome belonging to the other series; in other words, the chromosomes are present in biparental pairs (Montgomery, 1901; Sutton, 1902 and 1903; Wilson, 1912). "In the process known as synapsis, which takes place shortly before the last two cell-divisions concerned in the formation of the germ cells, the chromosomes do in fact unite in pairs, two by two. There is reason to believe that the two members of each pair are respectively of maternal and paternal derivation; and the probability of this view, first stated by Montgomery, has steadily increased." (Wilson, 1913). In one of these two final divisions of the germ cell cycle the double chromosome groups are reduced to single ones in preparation for the subsequent process of fertilization; this reduction is accomplished through the failure of the individual chromosomes to split in the process of mitosis. In this reducing division the two members of a synaptic pair are separated to pass into different daughter cells, but not necessarily

in such a manner that each daughter cell receives exclusively maternal or exclusively paternal chromosomes. The distribution of each pair of homologous chromosomes is entirely independent of that of every other pair, so that in the daughter cells combinations of non-homologous chromosomes occur regardless of their parental origin; each daughter cell usually receives a mixture of maternal and paternal chromosomes in varying proportions. Within the limits of the reduced number, any combination of the chromosomes furnished by the immature germ cell is possible in a daughter cell resulting from the reducing division, save that a single daughter cell cannot receive both members of a synaptic pair. Synapsis is thus explained by the provision which it makes that two homologous chromosomes shall in no case enter the nucleus of a single spermatozoon or mature egg (Sutton, 1902). The principle of random distribution or independent assortment of non-homologous chromosomes has been confirmed by the direct observations of Carothers (1913 and 1917), Wenrich (1915 and 1916) and others. The point that immediately concerns us here is the fact that in the reducing division the individuality of the germ-nuclei is usually lost, for it seldom happens that a single daughter cell, and consequently a single mature gamete, receives exclusively maternal or exclusively paternal chromosomes.

"Synapsis is not a haphazard junction of chromosomes, but an orderly union of elements of maternal and paternal origin, similar in size, in details of form, and probably also in function" (Kellicott, 1913, p. 294). The orderly processes of heredity as they exist today in biparental organisms would be impossible were the integrity of the maternal and paternal chromosome groups not maintained up to the time of synapsis. In *Cryptobranchus allegheniensis* and in some other forms this integrity is manifested at every step by a complete segregation of maternal and paternal chromosome *groups;* in other forms the independence of the chromosome groups is maintained in spite of the mingling of maternal and paternal chromosomes. The persistent individuality of the chromosomes is the important thing, and so long as this is maintained the germ-nuclei exist as actual

entities whether visibly separated or not. The distinction between the two classes of germ-nuclei—those that fuse at the time of fertilization and those that do not—is more apparent than real. The expression "individuality of the germ-nuclei" seems justified in either case, for individuality implies separableness as well as separateness (Conklin, 1916); but some may prefer to use the term "autonomy," which places the emphasis upon independence. Since attention has become focused upon the chromosomes, the expression "autonomy of the maternal and paternal chromosome groups" has often been used to indicate the duality of nuclear structure.

Where it exists, the separation of maternal and paternal chromatin-complexes into distinct groups within a single nucleus affords a striking exemplification of the deeper and more universal truth that each germ-nucleus is represented in its entirety in every cell, somatic as well as germinal, of a developing organism. In *Cryptobranchus allegheniensis* this dual structure of the nucleus is clearly visible in every cell of the early segmentation stages at least, so that here we have material for an ocular demonstration of a principle long ago foreseen by Huxley (1878), who wrote: "It is conceivable, and indeed probable, that every part of the adult contains molecules derived from both the male and the female parent; and that, regarded as a mass of molecules, the entire organism may be compared to a web of which the warp is derived from the female and the woof from the male."

Summary.

In the fertilization of the egg of *Cryptobranchus allegheniensis* the germ-nuclei do not fuse, and in the first cleavage mitosis each gives rise to a separate group of chromosomes whose descendants pass separately to the daughter-nuclei.

During the ensuing resting stage each germ-nucleus is represented by a structurally distinct vesicle. The separateness of the germ-nuclei is thus maintained throughout the entire nuclear cycle.

Throughout early cleavage the nuclear divisions are of the same duplex type, and the resting nuclei are always distinctly

double. The genetic continuity of each half of the double nucleus has been clearly traced to an advanced cleavage stage.

During late cleavage and in the early gastrula the nuclei are still typically double, but certain irregularities which tend to disguise the double structure occur with increasing frequency and the segregation of maternal and paternal chromatin cannot always be demonstrated.

The hypothesis of individuality of the germ-nuclei as applied to those species in which there is a mingling of maternal and paternal chromosomes is discussed, and supported by considerations regarding the persistent individuality of the chromosomes.

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Explanation of Plate I.

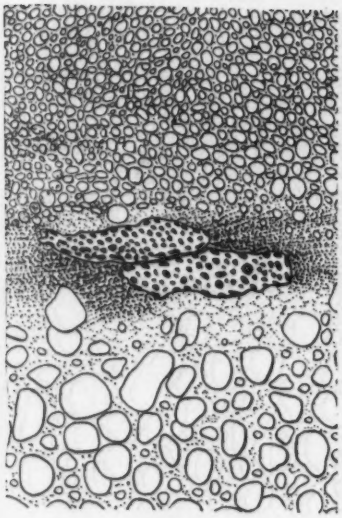
Cryptobranchus allegheniensis.

Fig. 1. Portion of a meridional section of a fertilized egg, showing the meeting of the sperm-nucleus and the egg-nucleus. Each germ-nucleus extends through four sections, of which an inner one is shown in the figure. The section passes midway between the two asters, which lie on opposite sides of the nuclei in a line at right angles to the plane of the section. The egg was fixed with bichromate-acetic formalin and stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 2. Meridional section of an egg killed twenty-two and one half hours after fertilization, showing the resting germ-nuclei. Each germ-nucleus extends through five sections, of which the middle one is here shown. The section passes midway between the two asters, which lie close to the apposed surfaces of the two nuclei in a line oblique to the plane of the section. Fixed with bichromate-acetic-formalin; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 3. Horizontal section through the resting germ-nuclei of an egg killed twenty-one and one half hours after fertilization. Nearly half of each nucleus is left in an adjacent section. Two asters are shown in the figure. Fixed with bichromate-acetic-formalin; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 4. Horizontal section through the resting germ-nuclei of an egg killed twenty-two and three fourths hours after fertilization. Each germ-nucleus extends through three sections, of which the middle one is here shown. Two asters are shown in the figure. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.



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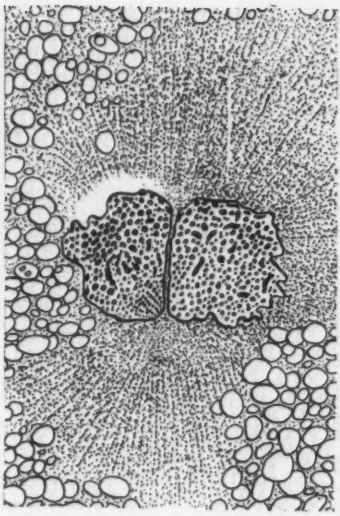

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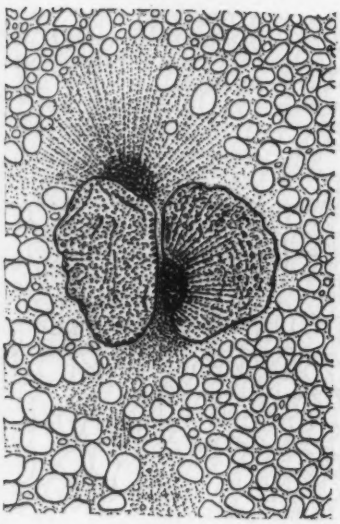

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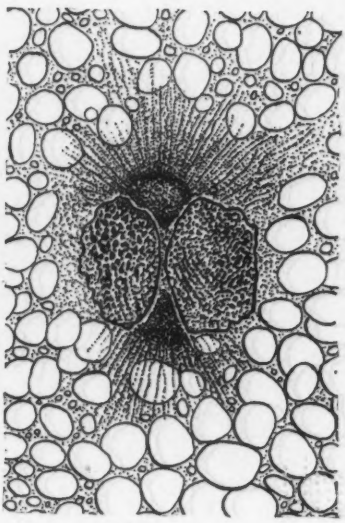

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Explanation of Plate II

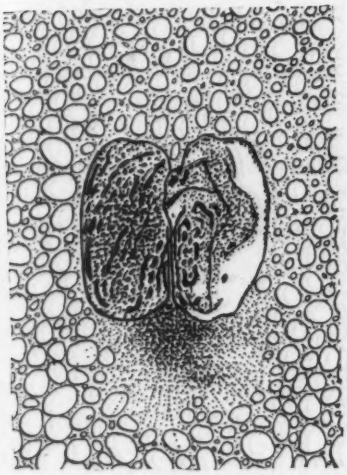
Cryptobranchus allegheniensis.

Fig. 5. Horizontal section of a fertilized egg showing the germ-nuclei preparing for the first cleavage mitosis. The germ-nucleus shown at the right is in a slightly more advanced phase than the other. A portion of each nucleus is left in an adjacent section. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 6. Horizontal section of an egg killed twenty-two and one half hours after fertilization, showing the germ-nuclei preparing for the first cleavage mitosis. The germ-nucleus shown at the right is in a more advanced phase than the other. About half of each nucleus is left in an adjacent section. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 7. Horizontal section through the first cleavage spindle (late prophase). The chromosomes are segregated into two groups, of maternal and paternal origin respectively. A very small portion of each group of chromosomes is left in each of the adjacent sections. Bichromate-acetic-formalin fixation; stained with iron-hæmatoxylin. × 400.

Fig. 8. Horizontal section of an egg killed twenty-six hours after fertilization, showing a late stage (early telophase) of the first nuclear division. In the distribution of chromatin to the daughter-nuclei, the segregation of maternal and paternal chromatin is maintained. A very small portion of the chromatin of each daughter-nucleus is left in an adjacent section. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric acid. × 400.



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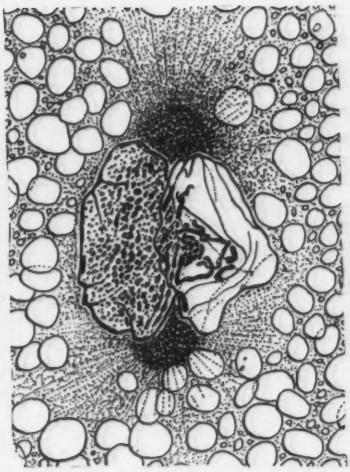

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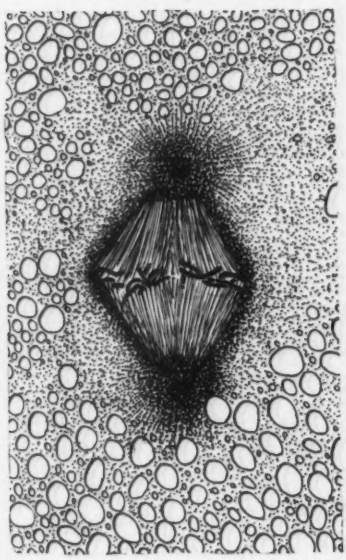

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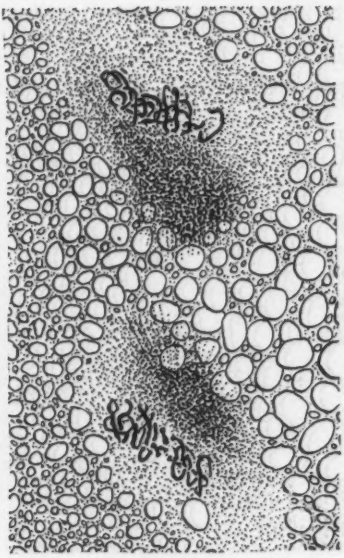

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Explanation of Plate III.

Cryptobranchus allegheniensis.

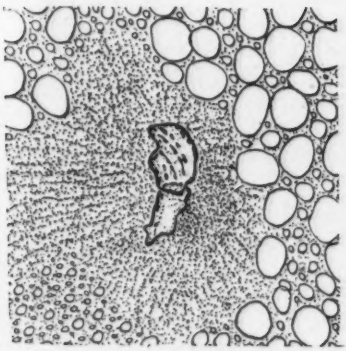
Figs. 9 and 10. Two successive vertical sections through a single daughter-nucleus of the first cleavage mitosis. The sections are nearly meridional, and pass very close to the other daughter-nucleus, hence at right angles to the plane of the first cleavage furrow which is not yet formed. The nucleus shown is almost entirely confined to the two sections. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric acid. × 400.

Fig. 11. Vertical section through a single daughter-nucleus of the first cleavage mitosis. The section is nearly meridional, and passes very close to the other daughter-nucleus, hence at right angles to the plane of the first cleavage furrow which is not yet formed. A considerable part of each nuclear vesicle is left in an adjacent section, and comparison with these other portions shows that the plane of contact of the two nuclear vesicles is oblique to the plane of the section. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 12. Vertical section showing a single daughter-nucleus of the first cleavage mitosis, taken at right angles to the plane of the first cleavage furrow which is not yet formed. The entire double nucleus is confined to this section. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 13. Vertical section, parallel to the newly-formed first cleavage furrow, through a single daughter-nucleus of the first cleavage division. Part of the nucleus is left in an adjacent section, where it is likewise distinctly double. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 14. Vertical section, parallel to the first cleavage furrow, through the other daughter-nucleus of the egg used for Fig. 13. Part of the nucleus is left in an adjacent section, where the two nuclear vesicles are more closely united. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.



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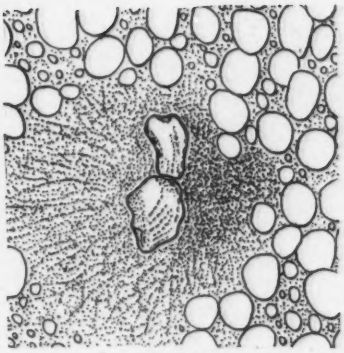

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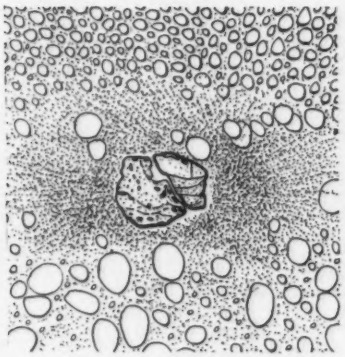

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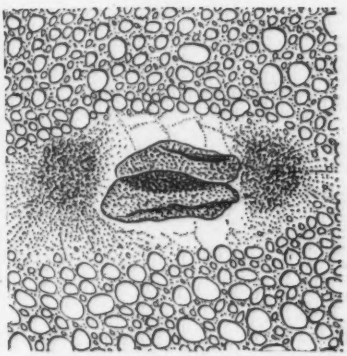

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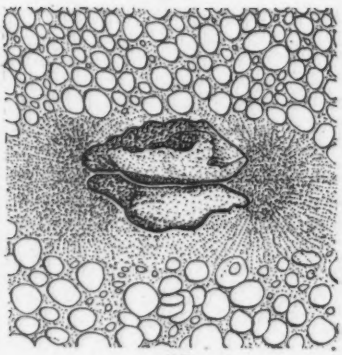

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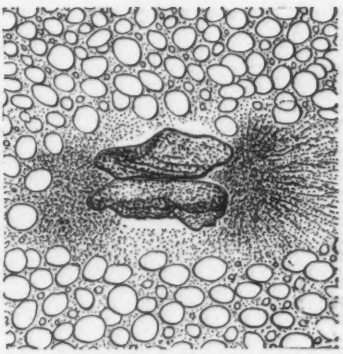

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BERTRAM G. SMITH.

Explanation of Plate IV.

Cryptobranchus allegheniensis.

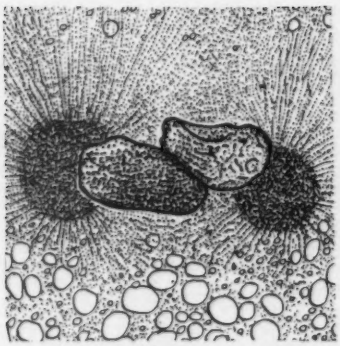
Figs. 15 and 16. Two successive horizontal sections showing a single nucleus rotating into position for the second cleavage mitosis; almost the entire nucleus is confined to these two sections. The newly-formed first cleavage furrow extends in a direction parallel to a line connecting the two asters. Fixed in bichromate-acetic-formalin; stained with iron-hæmatoxylin. × 400.

Fig. 17. Horizontal section through a single nucleus in position for the second cleavage mitosis; the greater part of the nucleus lies in this section. The newly-formed first cleavage furrow extends in a direction parallel to a line connecting the two asters. Fixed in Zenker's fluid; stained with iron-hæmatoxylin. × 400.

Fig. 18. Horizontal section through the other nucleus of the egg containing the nucleus shown in Fig. 17. Orientation as in the preceding figure. Stained with iron-hæmatoxylin. × 400.

Fig. 19. Horizontal section showing a single nucleus in position for the second cleavage mitosis; the entire nucleus lies in this section. The newly-formed first cleavage furrow extends in a direction parallel to a line connecting the two asters. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 20. Horizontal section through the other nucleus of the egg used for Fig. 19. A small part of one nuclear vesicle, and the greater part of the other, lies in an adjacent section. Orientation as in the preceding figure. Stained with borax-carmine Lyons-blue picric-acid. × 400.



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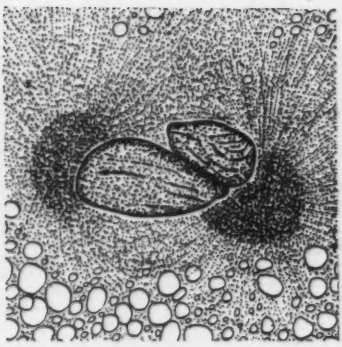

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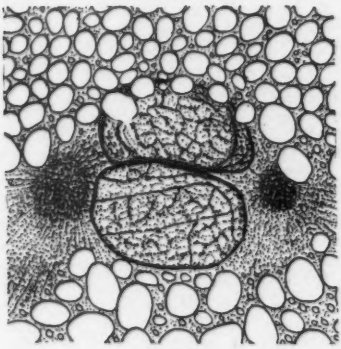

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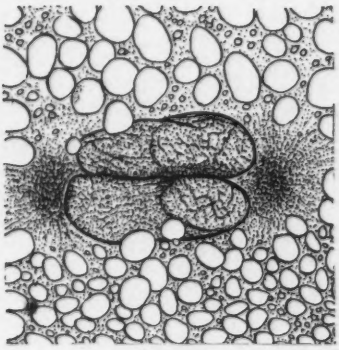

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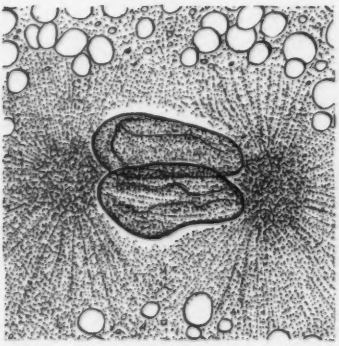

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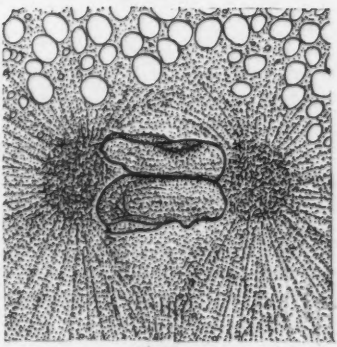

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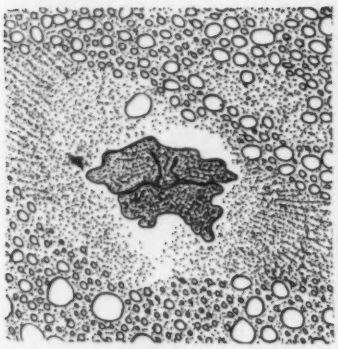
Explanation of Plate V.

Cryptobranchus allegheniensis.

Figs. 21 and 22. Two successive meridional sections through a single nucleus preparing for the second cleavage mitosis. The sections extend at right angles to the first cleavage furrow, and close to the other cleavage-nucleus. A considerable part of the nucleus here shown is left in an adjacent section where it is not so clearly separated into two nuclear vesicles. Fixed in bichromate-acetic-formalin; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 23. Horizontal section through a single nucleus preparing for the second cleavage mitosis; the nucleus is almost entirely confined to this section. The first cleavage furrow extends in a direction nearly parallel to a line connecting the two asters. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.

Figs. 24, 25 and 26. Three successive horizontal sections through a single nucleus entering upon the second cleavage mitosis; all but a very small portion of the nucleus is included in these sections. The first cleavage furrow extends in a direction nearly parallel to a line connecting the two asters. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.



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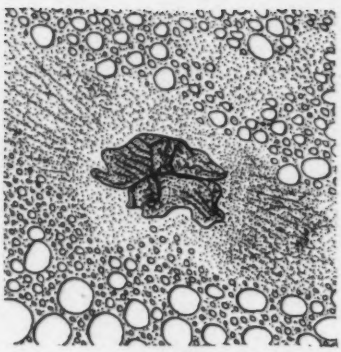

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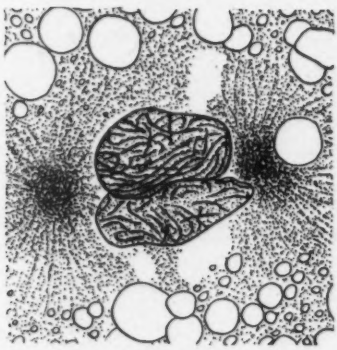

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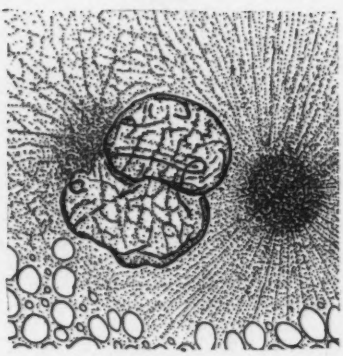

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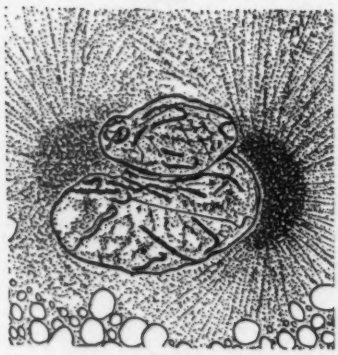

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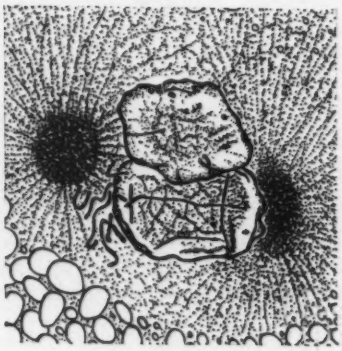

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BERTRAM G. SMITH.

Explanation of Plate VI.

Cryptobranchus allgeheniensis.

Fig. 27. Horizontal section through a nucleus in a late prophase of the second cleavage mitosis. A small portion of each group of chromosomes is left in each of the adjacent sections. The axis of the spindle lies in a direction nearly parallel to the plane of first cleavage. Fixed with bichromate-acetic-formalin; stained with borax-carmine Lyons-blue picric-acid. × 400.

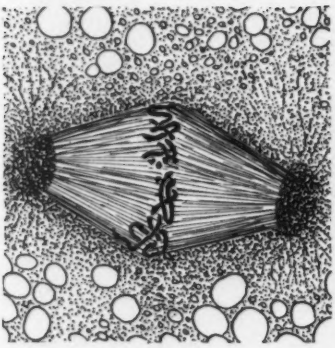
Fig. 28. Horizontal section through one of the daughter-nuclei in a late stage (early telophase) of a second cleavage mitosis. Orientation as in the preceding figure; the second cleavage furrow is not yet formed. Fixed in Zenker's fluid; stained with iron-hæmatoxylin. × 400.

Fig. 29. Horizontal section through one of the daughter-nuclei of a second cleavage mitosis; early resting stage. Part of the nucleus is left in an adjacent section where it is likewise distinctly double. The plane of apposition of the two nuclear vesicles is parallel to the first cleavage furrow, and at right angles to the second cleavage furrow which is beginning to form. Bichromate-acetic-formalin fixation; stained with iron-hæmatoxylin. × 400.

Fig. 30. Horizontal section through one of the daughter-nuclei of a second cleavage mitosis; resting stage. A portion of the nucleus is left in each of the adjacent sections where it is likewise distinctly double. The plane of apposition of the two nuclear vesicles is parallel to the plane of first cleavage, and at right angles to the beginning second cleavage furrow. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 31. Vertical section through one of the daughter-nuclei of a second cleavage mitosis; resting stage. The section is taken parallel to the plane of the second cleavage furrow which has just begun to form. Part of the nucleus is left in an adjacent section, where it is likewise distinctly double. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 32. Horizontal section through a nucleus rotating into position for the third cleavage mitosis; almost the entire nucleus is confined to this section. The second cleavage furrows are present and extend nearly to the equator of the egg. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.



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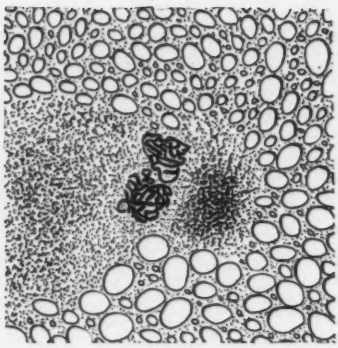

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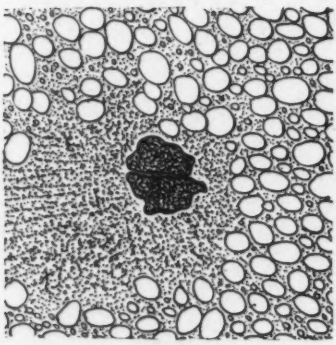

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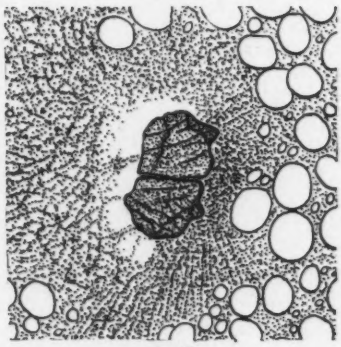

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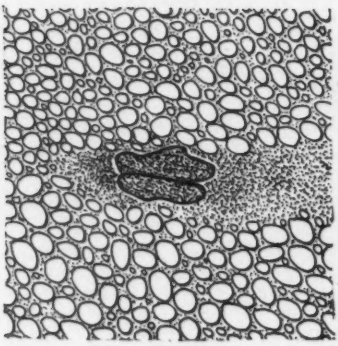

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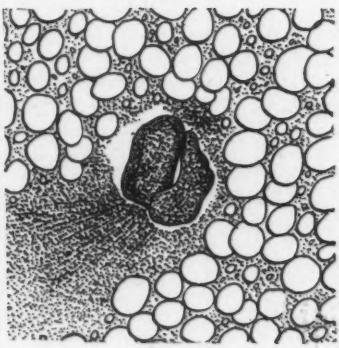

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BERTRAM G. SMITH.

Explanation of Plate VII.

Cryptobranchus allegheniensis.

Fig. 33. Horizontal section through a resting nucleus in position for the third cleavage mitosis. The plane of apposition of the two nuclear vesicles is nearly parallel to the *second* cleavage furrows. A considerable part of the nucleus is left in an adjacent section, where it is likewise distinctly double. Fixed with bichromate-acetic-formalin; stained with borax-carmine Lyons-blue picric-acid. × 400.

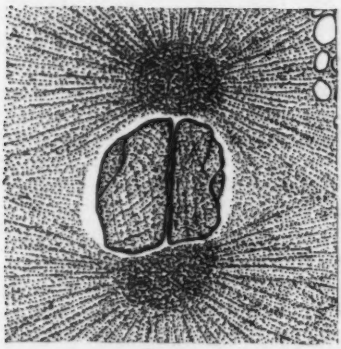
Fig. 34. Horizontal section through a nucleus in position for the third cleavage mitosis. A line connecting the two asters is nearly parallel to the second cleavage furrow. Part of the nucleus is left in an adjacent section, where it is likewise distinctly double. Fixed with bichromate-acetic-formalin; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 35. Vertical section through a nucleus entering upon the third cleavage mitosis. Part of the nucleus is left in each of the adjacent sections, where it is likewise distinctly double. Fixed with bichromate-acetic-formalin; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 36. Horizontal section through a nucleus in the late prophase of the third cleavage mitosis. A line connecting the two asters extends nearly at right angles to the first cleavage furrow. Fixed with bichromate-acetic-formalin; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 37. Horizontal section through a daughter-nucleus of a third cleavage mitosis. The two nuclear vesicles remain almost in the position in which they were formed, but two asters are present and these have taken up a position preparatory to the fourth cleavage mitosis. A line connecting the two asters extends in a direction almost parallel to the adjacent newly-formed third cleavage furrow which is vertical and intersects a second furrow almost at right angles. Fixed in bichromate-acetic-formalin; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 38. Horizontal section through a nucleus in position for the fourth cleavage mitosis. A line connecting the two asters extends parallel to the adjacent third cleavage furrow and almost parallel to the first furrow. Fixed in bichromate-acetic-formalin; stained with borax-carmine Lyons-blue picric-acid. × 400.



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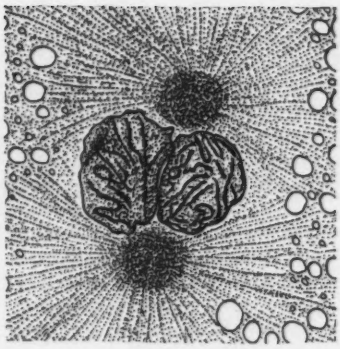

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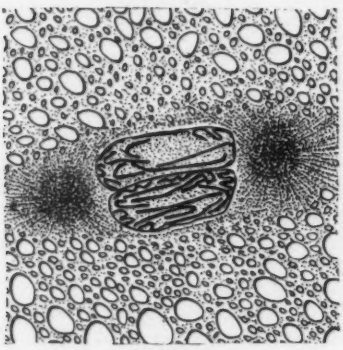

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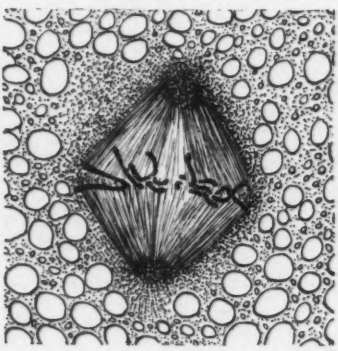

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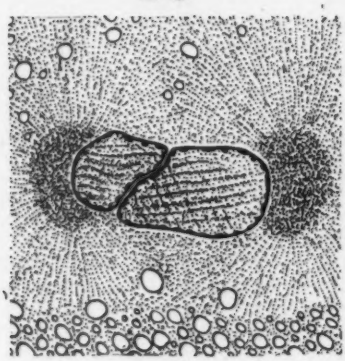

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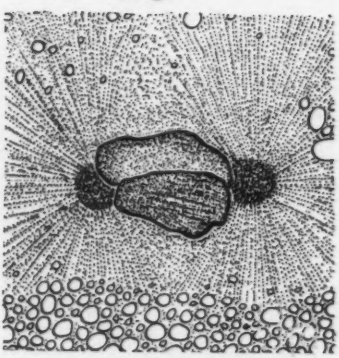

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BERTRAM G. SMITH.

Explanation of Plate VIII.

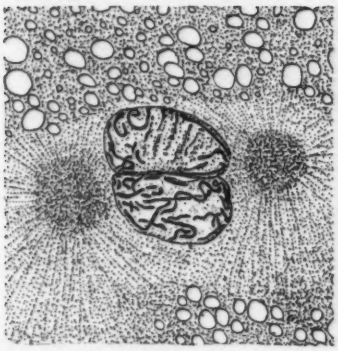
Cryptobranchus allegheniensis.

Figs. 39 and 40. Two successive vertical sections through a nucleus preparing for the fourth cleavage mitosis; almost the entire nucleus lies in these two sections. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.

Fig. 41. Another nucleus found in the section used for Fig. 39; early prophase of the fourth cleavage mitosis. × 400.

Fig. 42. Vertical section through a nucleus in the metaphase of the fourth cleavage mitosis. The fourth cleavage furrow has not yet been formed. Bichromate-acetic-formalin fixation; stained with iron-hæmatoxylin. × 400.

Figs. 43 and 44. Two successive horizontal sections through a newly-formed daughter-nucleus of a fourth cleavage mitosis. The fourth cleavage furrow is newly formed. Bichromate-acetic-formalin fixation; stained with borax-carmine Lyons-blue picric-acid. × 400.



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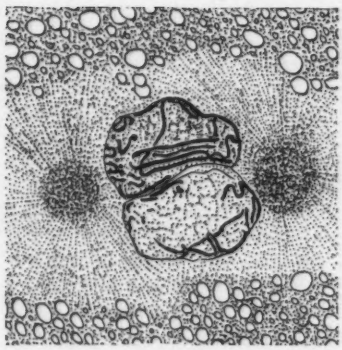

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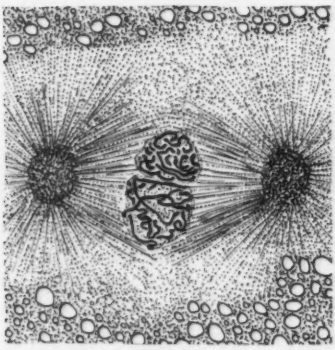

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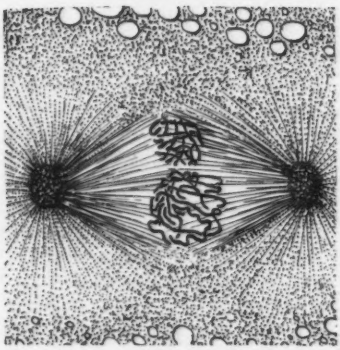

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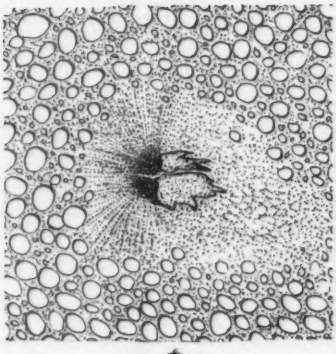

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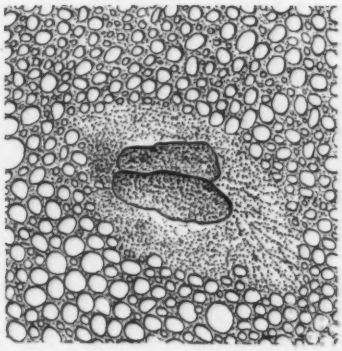

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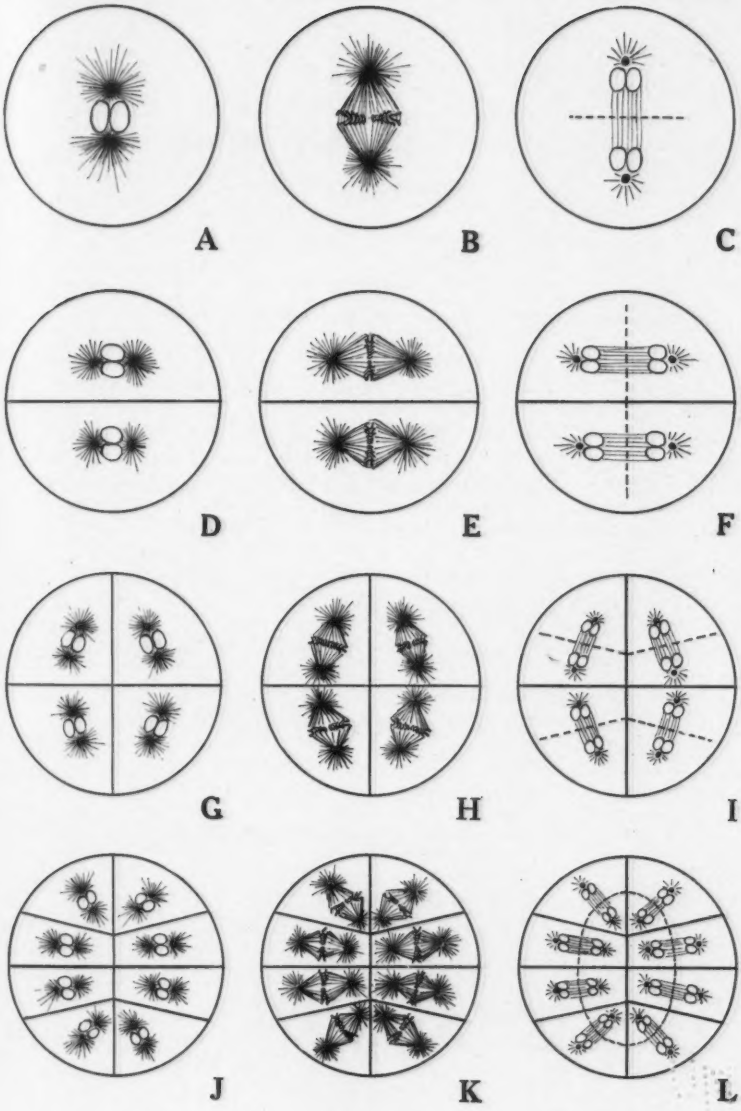
BERTRAM G. SMITH.

Explanation of Plate IX.

Cryptobranchus allegheniensis.

Fig. 45. Diagrams illustrating the orientation of the germ-nuclei during the first four cleavage divisions. All the diagrams represent horizontal sections, and the germ-nuclei are assumed to lie always in the same horizontal plane. The size of the nucleus is exaggerated in proportion to the size of the egg. Dotted lines represent beginning cleavage furrows; solid straight lines represent cleavage furrows which have cut to the level of the nuclei. The number of chromosomes formed from each germ-nucleus is assumed to be six, and this is probably the correct number.

A. Germ-nuclei and asters in position for the first cleavage mitosis.
B. First cleavage mitosis.
C. Newly-formed daughter-nuclei of the first cleavage mitosis.
D. Nuclear vesicles and asters in position for the second cleavage mitoses.
E. Second cleavage mitoses.
F. Newly-formed daughter-nuclei of the second cleavage mitoses.
G. Nuclear vesicles and asters in position for the third cleavage mitoses.
H. Third cleavage mitoses.
I. Newly-formed daughter-nuclei of the third cleavage mitoses.
J. Nuclear vesicles and asters in position for the fourth cleavage mitoses.
K. Fourth cleavage mitoses.
L. Newly-formed daughter-nuclei of the fourth cleavage mitoses.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